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(54) Title: NON-DENDRITIC BACKBONE PEPTIDE CARRIER

(57) Abstract

The present invention relates to a non-dendritic peptide designed for use as a carrier of an immunogenic substance and/or an immune mediator, a construct of said carrier carrying an immunogenic substance and/or an immune mediator, a process for the preparation of immunogens with high and predictable immunogenicity which comprise said non-dendritic peptide carrier, use of such immunogens for the production of vaccines and vaccines comprising an immunogenic substance and/or an immune mediator on the peptide carrier. The invention also relates to diagnostic or therapeutic embodiments using the non-dendritic peptide carrier, to diagnostic or therapeutic compositions and to methods for the use thereof in diagnosis of diseases and pregnancy as well as in therapy. The non-dendritic peptide carrier according to the invention comprises 10-50 amino acids capable of forming a secondary structure in a benign buffer after liberation from the solid phase.

NON-DENDRITIC BACKBONE PEPTIDE CARRIER

The present invention relates to a non-dendritic peptide
designed for use as a carrier of an immunogenic substance
and/or an immune mediator, a construct of said carrier car-
5 rying an immunogenic substance and/or an immune mediator, a
process for the preparation of immunogens with high and
predictable immunogenicity which comprises said non-dendritic
peptide carrier, use of such immunogens for the production of
vaccines and vaccines comprising an immunogenic substance
10 and/or an immune mediator on the peptide carrier. The in-
vention also relates to diagnostic or therapeutic embodiments
using the non-dendritic peptide carrier to diagnostic or
therapeutic compositions and to methods for the use thereof
in diagnosis of diseases and pregnancy as well as in therapy.

15 The non-dendritic peptide carrier is coupled by its C-ter-
minus through a specifically cleavable "linker" to a solid
phase forming a solid phase complex for the preparation of
the carrier peptide synthesizing or coupling the immunogenic
substance thereon before the non-dendritic peptide carrier is
20 released from the solid phase.

BACKGROUND

In WO 95/31480, an antiparallel "coiled-coil" heterodimer has
been used as an immunogen carrier. By use of heterodimers,
the preparation of a composition comprising different
25 immunogenic target molecules substances is controlled. The
preparation involves mixing two different monomers on which
the desired two different target molecules are attached, one
on each type of monomer, respectively. As each type of mon-
omer is designed not to form homodimers, only heterodimer
30 formation is favoured. The heterodimers obtained comprise
both of the desired two different immunogenic target mol-
ecules, one on each monomer. Accordingly, the immunogen
composition comprises different immunogen molecules which are
coupled on the peptide carrier by conventional coupling

methods and only one immunogenic substance on each monomer is shown. This self-assembling system thus assures the combination of two different substances in the same dimer, e.g. a B-cell- and a T-cell-epitope, respectively. Conformational

5   stabilization was not investigated and the problem of dissociation at low concentrations was not addressed. Subunit peptides are between 21 and 70 amino acids.

In WO 95/11998, structured peptide libraries are shown which are based on the antigenic variations in various pathogenic

10   microorganisms or on a repertoire of T-cell stimulating sequences. The aim is to achieve a broad reactivity or immunogenicity both with respect to the variants of the microorganism and with respect to the host population. The library is synthesized simultaneously in a single peptide synthesis

15   comprising adding a mixture of reactants representing a distribution corresponding to the desired distribution of the final peptide population representing the library. The library is synthesized using solid phase, however, no specific backbone structure is disclosed apart from a con-

20   ventional lysine containing derivatizable polymer.

In WO 94/29332, an antiparallel intramolecular "coiled-coil" monomer has been used as an immunogen carrier in which the immunogenic substance in the form of peptide sequences are interposed in the linear peptide forming the monomer whereby

25   the total immunogen composition constitutes a pure peptide. Accordingly, a carrier is described which is stabilized by an intramolecular, antiparallel arrangement of two amphipathic α-helices combined by a turn, the stability of which is not concentration-dependent as is the case with α-helix bundles.

30   The structure is not a scaffold designed for carrying multiple immunogenic substances but rather a template for a stable design. The whole peptide can be between 32 and >200 residues. Branch-peptide attachment is not mentioned. No immunization examples are presented.

Cyclic or constrained scaffolds used for attachment of pep-
tides to generate "template assisted synthetic proteins"
("TASP"s) are known (Tuchscherer 1993). These scaffolds were
specifically designed to position the attached peptides on a
5   rigid template to enhance the conformational traits of these
peptides and has been convincingly shown to do just that by
circular dichroism measurements, at least with α-helical
peptides. Immunization with TASPs in the presence of adjuv-
ants seems to generate protein cross-reacting antibodies
10   without further conjugation to other carriers. Their syn-
thesis involves a solid-phase synthesis of the scaffold,
followed by partial deprotection and synthesis of the
attached peptides, followed by cleavage and most often
cyclization and extensive purification by HPLC.

15   Also chemoselective coupling of pre-synthesized peptide
thioesters to a bromoacetylated scaffold has been described
(WO 95/04543). Different types of structures can be coupled
by using orthogonal chemistry (Tuchscherer 1993). Structures,
including mixed structures with T-cell and B-cell peptides in
20   various ratios and their use as immunogens have also been
reported (Kaumaya 1993)). The scaffold therein consists of
two short ß-strands connected by a turn and immunizations
were performed with Freund's complete adjuvant leading to
antibodies reacting with the peptides and the corresponding
25   native protein. No direct evidence is presented as to the
support of structure in the attached peptides, and immuniz-
ations are generally performed in the presence of adjuvants.

GB 2 282 813 A describes a scaffold which is cyclised by a
thioether linkage and contains lysine residues providing
30   amino groups as attachment points, whereupon cyclic B-cell
epitopes as well as linear peptide T-cell-stimulating
antigens can be coupled. The scaffold itself is synthesized,
cyclised, and purified, and, subsequently, purified peptide
antigens, specifically cyclized peptide B-cell antigens and
35   linear T-cell antigens are coupled by chemical means. Also
carbohydrates can be coupled and groups with adjuvant activ-

ity, e.g. tripalmitate-Cys-structures can be included. The
products are not readily soluble and are administered with
liposomes or emulsified for immunizations. There is no data
concerning structure support nor is anything presented with
5 respect to ability to induce protein-cross-reacting anti-
bodies. There is no mentioning of synthesis on solid-phase
bound scaffold.

In US Patent No. 5229490, synthetic peptide scaffolds are
disclosed having a "dendritic" construction. Multiple antigen
10 peptide systems are described in which a large number of
antigens are bound to the functional groups of a dendritic
core molecule also called "multiple antigenic peptides"
("MAPs"). The antigen-presenting dendrimer is generated by
chemical synthesis of a number of "layers" on both amino-
15 groups of α- and ϵ-unprotected lysine, conjugated by its C-
terminal to a solid phase, yielding, typically 4 or 8 deri-
vatizable amino groups per one residue of solid-phase-
attached lysine. These groups may be derivatized to present
other functionalities and/or spacer-moieties (see WO 92/18528
20 and Tam 1995). By attaching antigenic peptides to the outer
amino groups of the solid-phase bound MAP-core, followed by
cleavage of the completed structure, a so called high-density
presentation is achieved with a minimal "core" carrier
structure, each lysine moiety in the outer layer carrying two
25 antigenic peptides. Such MAPs may be highly immunogenic, at
least when injected in the presence of adjuvants like
Freund's incomplete adjuvant and aluminium hydroxide yielding
antibodies exclusively directed against the antigenic peptide
"branches". In some cases, however, these antibodies, even if
30 having high affinity to the MAP-bound antigenic peptides, are
not very reactive with the native structure from which the
peptide is derived (see e.g. Briand 1992). In addition,
indications prevail that the number of "branch"-peptides
influences the structure of the individual branches (see e.g.
35 Francis 1991), and no facts are presented as to whether the
desired retention of the peptide structure in the antigenic
peptide branches is present. Their chemical synthesis is

tedious, as the closely situated branch-peptides tend to aggregate. Therefore, protocols generally recommend an "indirect approach", in which the peptide antigens are coupled as pre-synthesized entities (Tam 1993). However, all

5 the problems of no relation to the synthesis are still present.

MAPs have also been found to be difficult to characterize and purify by high pressure liquid chromatography (HPLC). Accordingly, MAPs are normally used for immunization in an essenti-

10 ally unpurified state (apart from e.g. a desalting step or a crude gel filtration step) (see e.g. Tam 1993), deflating the advantage of using a chemical method to produce the conjugate.

In order to improve vaccines and immunizations, an important

15 goal is to refine the antigen used and especially to ensure its purity and its chemical definability while still retaining its ability to evoke a beneficial immunological response. However, the known carrier peptides and production of such peptides and immunogenic compounds are inadequate in this

20 respect. Generally, liposome-formulation or formulation with oil-in-water adjuvants is needed to create an adequate antibody response.

Well-established methods for increasing the immunogenicity of peptides exist (see e.g. Plaue 1990). Conventional methods

25 generally involve the conjugation of the peptide by chemical means to a carrier molecule (see e.g. van Regenmortel 1988). The chemical conjugation methods, well-known to those skilled in the art, comprise coupling with glutaric aldehyde (coupling to amino- and thiol groups), carbodiimides (coupling to

30 amino groups), m-maleimide benzoyl-N-hydroxysuccinimide esters (coupling from amino- to thiol-groups), and coupling carbohydrates through oxidation followed by reaction with primary amino groups and reduction as well as coupling thiols by specific reagents (see e.g. van Regenmortel 1988). The

35 carrier is typically a naturally-derived protein, as e.g.

albumin, ovalbumin, purified protein derivative (PPD) from *M. tuberculosis*, keyhole limpet hemocyanin, avidin, diphtheria toxoid, tetanus toxoid, etc.

The immunization schedule often and typically entails mult-
5 iple sequential injections of the peptide-carrier conjugate intramuscularly, subcutaneously or intradermally together with adjuvants including Freund's complete adjuvant, Freund's incomplete adjuvant, Emulsigen^R, Titermax^R, aluminium hydrox- ide, etc. The only adjuvant at the present date allowed for
10 human use is aluminium hydroxide. A rather new type of adjuvant is the immunostimulating complex ("Iscom") (Morein 1984) in which a combination of amphiphilic antigen, quil A, cholesterol, lipid and phosphatidylcholine leads to the formation of small (35 nm in diameter), highly immunogenic
15 particles in which the antigen is embedded in the membrane turning its hydrophilic parts outwards. This method has especially shown to work with viral antigens. Iscoms may be used as a peptide carrier/adjuvant by conjugating the pep- tides by chemical methods to a preformed Iscom containing a
20 suitable protein (Larsson 1993). Another type of carrier functioning as an adjuvant and usable with synthetic peptides is liposomes containing lipid A and a reactive group for chemical coupling of peptides (Friede 1993).

Methods for producing immunogenic peptides by carrier-coupl-
25 ing, however, are largely empirical, and, normally, it is not possible to predict the optimal carrier, conjugation method, and conjugation density for the induction of a desired immune response. Important parameters such as the orientation by which the peptide antigen is coupled to the carrier are not
30 controlled, although of major importance for the produced antibodies. Accordingly, the problems, which are overcome according to the present invention, include problems of chemical definability as follows:

1. The ratio of peptide to carrier is only measured with some
35 difficulty, e.g. by amino acid analysis, radioactivity count-

ing or by estimates based on accurate molecular weight deter-
minations.

2. With most chemistries, the peptide is coupled to the
carrier in random orientation and by the attachment of a

5   varying number of functional groups along the peptide chain
possibly leading to the destruction of the charge-distrib-
ution and structure of the peptide.

3. Peptide-peptide- and carrier-protein - carrier-protein
polymer-formation can only be excluded by careful case-to-

10   case optimization of reaction conditions possibly in combin-
ation with post-reaction clean-up, e.g. by size-exclusion
chromatography.

4. Naturally derived carrier proteins are not chemically
totally characterized.

15   5. The conjugation procedure increases the time needed to
produce the peptide-immunogens, adding a post-synthesis step,
and also increasing loss of material.

6. Antibodies formed against the carrier protein and the
conjugation group may cause problems.

20   7. Naturally derived carrier proteins of "immunization"
quality are quite expensive.

Finally, as a rule, naturally derived (or recombinant) car-
rier-proteins are not allowed for administration in humans as
immunogens.

25   According to the present invention, the drawbacks mentioned
above can be circumvented by using totally synthetic immuno-
gens, e.g. synthetic peptides coupled to a synthetic peptide
carrier, provided that this carrier presents the peptide
immunogen at high density, supplies the necessary T-cell-
30   epitopes, and supports the conformation of the peptide.

Benefits include the possibility of direct, sequential syn-
thesis on a solid-phase bound peptide carrier (no need for
post-synthesis operations), and a greater variety of chemical
methods being amenable, e.g. allowing different substances to
5 be coupled by orthogonal chemistries and allowing control of
orientation of the peptide. Also, the development of anti-
bodies to carrier proteins <u>and</u> coupling groups is avoided.

The chemical synthesis of peptides by solid-phase synthesis
is well known (Merrifield 1963, Atherton 1978). Peptides are
10 synthesized sequentially from the C-terminus, which is bound
to an insoluble solid phase by a specifically cleavable
chemical "linker". After completion of the synthesis, the
peptide is liberated by cleavage of the linker. Four groups
of chemistries are combined, the first one being used for
15 activating the α-carboxylic group of the amino acids to be
coupled, the two other ones being used for protecting α-amino
groups and side-chain groups, respectively, and the fourth
one controlling the lability of the peptide-to-solid phase
linker.

20 In one embodiment, the present invention relates to a
copolymer consisting of a conventional solid-phase peptide
synthesis polymer to which is coupled an oligopeptide cont-
aining a number of freely accessible functional groups on
which additional peptides can be synthesized or coupled or
25 other entities can be coupled. Peptide synthesis on the
copolymer can be performed by conventional methods of solid-
phase synthesis. After synthesis or coupling, the whole
complex can be cleaved and brought into solution and can be
purified and analysed by conventional means. Additionally,
30 the coupled peptides can be sequenced. The complex presents
the coupled peptides very efficiently, increasing the immun-
ogenicity of the coupled peptides considerably. The complex
can thus be used as an immunization means, either alone or in
combination with Iscom-forming agents or with other adjuv-
35 ants.

For a full chemical definition, it is preferable, if pos-
sible, to synthesize the appropiate antigen chemically,
rather than to derive the antigen from natural sources. To be
feasible, such a synthesis should be economical and lead to
practical amounts of the antigen with the desired purity.
Such easily and economically synthezisable antigens include
short peptides, i.e. peptides comprising fewer than approxim-
ately 30 amino acid residues.

The use of short synthetic peptides as antigens in vaccines
has a number of advantages, including increased safety,
increased reproducibility, low production costs, and possib-
ility of choosing non-mutating epitopes (see e.g. Shinnick
1983). Antibodies generated against synthetic peptides are
directed against predetermined parts of a protein; this is
not possible by other methods. Antibodies may also be
directed against non-immunogenic parts of proteins or against
non-peptide antigens, e.g. carbohydrates or haptens. Cancer,
viral, including HIV, bacterial, and parasitic infections
have been investigated with respect to peptide-based vac-
cines.

In a basic research setting, peptides can be used for the
production of antibodies against non-identified proteins of
which the gene sequence is known and for mapping sites in a
protein. In the vaccine field, totally synthetic vaccines are
advantageous for several reasons including economy, high
batch-to-batch reproducibility, increased stability, ex-
clusion of impurities, and the above-mentioned possibility of
obtaining a "clean" response only directed against the crit-
ical part(s) of the pathogen. Such synthetic construct also
have many application as antigens in diagnostic assays and as
therapeutics.

A neutralizing response against a whole microorganism raised
by a single short synthetic peptide has, however, been demon-
strated in only a few special cases. This is due to the fact
that peptides, as a rule, are not immunogenic, i.e. not able

to induce a substantial immune response by themselves. Short
peptides are not believed to be big enough to ensure a full
immune response (humoral as well as cellular response)
because the major histocompatibility complex does not bind
peptides which do not have the right sequence and which lacks
a synergistic or cooperative effect seen with bigger immun-
ogens presenting several epitopes to the host cell at the
same time (multivalency). If antibodies are obtained, they
typically have low affinities, as the conformation of short
peptides is not defined very well in aqueous environments;
therefore, the resulting antibody is not selected to the
constricted conformer of the peptide in its polypeptide-
enclosed, natural state. This leads to an undesirable low
degree of cross-reactivity between an anti-peptide antibody
and the corresponding protein detrimental to most vaccine-
applications where the antibody-response should neutralise an
invading pathogen, e.g. by binding to a specific toxin, or by
binding to surface-molecules implicated in binding of the
pathogen to host tissue or by binding to a pathogen-specific
antigen preparing the microorganism for phagocytosis.

The design of short peptides (< ca. 30 amino acids) which
retain structure in aqueous environment is a major task. The
structure of short peptides may be increased by chemical
derivatizations restricting the conformational freedom of the
peptide, e.g. by cyclization or polymerization (see e.g Robey
1992 and Gilon 1991) or by the inclusion in the chain of
structure-nucleating substances, eg. turn- or α-helix-
inducers (e.g. Kahn 1993, Unson 1984, Kemp 1990, Hinds 1991,
Días 1993 and Bambino 1994) or chelated metal-ions (e.g.
Regan 1995). However, the only single unmodified peptide
structure capable of retaining conformation in water is the
amphipathic α-helix (Mant 1993) which is stabilized by ag-
gregation into homo- or heterodimers or oligomers. An amphi-
pathic helix has a hydrophobic side occupying one half face
of the helix along its axis and a hydrophilic side on the
other half. Amphipatic α-helices typically combine in
"bundles" shielding the hydrophobic face and exposing the

hydrophilic side to form parallel or antiparallel homodimers (Zhu 1993) or heterodimers and oligomers (Zhu 1992).

In the present context, the definitions below are used:
A peptide carrier is a substance comprising a peptide that serves as a carrier of one or more moieties which may themselves be peptides, each moiety being chemically coupled to said carrier peptide through a linkage involving an amino acid side chain of the carrier peptide.

A non-dendritic peptide carrier is a peptide carrier that does not contain any double-derivatizable building blocks which are substituted in any of the derivatization groups with another similar or different double-derivatizable building block, said non-dendritic carrier peptide further providing at least two derivatizable functional groups.

In the description of the non-dendritic peptide carriers and the derivatized non-dendritic peptide carriers, the following terms are used:

Backbone peptide denotes the non-dendritic peptide carrier itself. Branch-peptide or "branch-moiety" denotes the peptide or moiety coupled to the non-dendritic peptide carrier which thereby is derivatized. Attachment point denotes the functional groups in the non-dendritic peptide carrier available for derivatization.

A lipidic moiety is defined as an alkyl or alkenyl fatty acid bound by its carboxylic function as an amide or an ester.

A solid-phase linker for peptide synthesis is a molecule with at least two functional groups, one used for providing a stable covalent linkage to the solid phase polymer, and the other one used for attaching the C-terminal amino acid by a stable covalent linkage during peptide synthesis, said amino acid attaching linkage being stable to the peptide synthesis conditions but having a defined lability to specific chemical

treatments by which the produced peptide can be liberated
either as a free acid or a C-terminally modified peptide,
depending on the chemical treatment.

An <u>antigen</u> is a substance which is reactive with a specific
antibody or T-cell.

An <u>immunogen</u> is a substance capable of inducing an antigenic
response including antibodies and a T-cell response.

An <u>immune mediator</u> is a substance which is capable of regul-
ating the activity of the immune system. Such a regulating
effect may refer to an activation of the immune system as
well as a down regulation of the immune system. The term
<u>immunomodulator</u> is used interchangeably with the term immune
mediator.

An <u>adhesion molecule</u> is derived from a cell surface and is
capable of binding to another specific cell surface derived
molecule. Selectins and CAM molecules are examples of
adhesion molecules.

A <u>T-cell stimulatory peptide, -antigen, -protein and a T-cell
antigen</u> are used interchangeably and denote a molecule that
is capable of stimulating the proliferation of a specific T-
lymphocyte clone in the immunized host resulting in a T-
lymphocyte-mediated immunogenic response, as well as a mol-
ecule that is reactive with a specific T-lymphocyte clone.

A <u>B-cell antigen</u> is a molecule that is reactive with a spec-
ific B-lymphocyte clone or that elicits a B-lymphocyte-medi-
ated immunogenic response in a subject or test animal.

A <u>benign</u> buffer is a physiologically compatible aqueous
buffer with a pH between about 6 and about 8 and a salt
concentration between about 50 mM and about 500 mM, pre-
ferably between about 100 mM and about 200 mM.

Peptide and polypeptide are used interchangeably to denote a polyamide chain of amino acids from 2 amino acids to 100 or more amino acids long.

A stabilized or supported secondary structure is defined as a preferred conformational state of the peptide in question, obtained by restricting the rotation of the peptide bonds comprising the peptide into a relatively fixed and well-defined structure by a "secondary structure supporting moiety".

Peptide amino acid residues are always counted from the N-terminus, the N-terminus being number 1.

Sequences for peptides and polypeptides are given in the order from the amino terminus to the carboxyl terminus.

All amino acids are represented by standard one- or three-letter abbreviations as commonly used in the art.

As used herein, natural amino acids are the 20 amino acids, either in L- or D-forms commonly found in proteins, e.g. alanine, aspartic acid, asparagine, arginine, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, tryphtophan, and alanine.

Unnatural amino acids include, but are not limited to, D- and L-forms of epsilon-aminohexanoic acid, gamma-amino butyric acid, alpha-aminobutyric acid, alpha-aminoisobutyric acid, alpha-aminoadipic acid, allo-threonine, allo-isoleucine, 7-aminoheptanoic acid, norleucine (2-aminohexanoic acid), norvaline (2-aminopentanoic acid), delta-aminovaleric acid (5-aminopentanoic acid), 11-amino-undecanoic acid, t-Butyl-alanine, t-Butyl-glycine, gamma-carboxyglutamic acid, citrulline, homocysteine, homocitrulline, homoarginine, homophenylalanine, delta-hydroxylysine, 4-hydroxy-proline,

isoasparagine, isoglutamine, isoserine, penicillamine, phe-
nylglycine, thyroxine, ornithine, and the like.
The term amino acid denotes both groups.

A PNA or peptide nucleic acid substance is a molecule con-
sisting of a peptide chain of unnatural amino acids selected
from amino acids containing naturally occurring DNA- or
nucleobases in their side chains (see WO 95/01369).

DESCRIPTION OF THE INVENTION

It is an object of the present invention to provide a general
non-dendritic peptide carrier that provide a high-density
presentation of bioactive moieties in a predefined particular
arrangement, orientation and stoichiometry. The core peptide
carrier is not immunogenic and the construct is chemically
unambigous. Coupled antigens are presented very efficiently,
increasing their immunogenicity considerably. The complex can
thus be used as an immunization means, either alone or in
combination with Iscom-forming agents or with other ad-
juvants.

The defined oligopeptide (the non-dendritic peptide carrier)
of the present invention comprises more than one accessible
functional group and is furthermore derivatized in one or
more locations by a strongly hydrophobic moiety, preferably a
long-chain fatty acid like palmitic or myristic acid, and
bound through its C-terminal amino acid to a polymer called a
solid phase. On said oligopeptide, antigenic entities can be
coupled by chemical methods by direct synthesis or by coupl-
ing "en bloc", leading to a branched complex that can be
cleaved in its intact entity from the solid phase and can be
characterized and if necessary purified therefrom by standard
methods.

The invention further comprises a method for the synthesis of
the oligopeptide as well as the synthesis of branched com-
plexes.

The non-dendritic peptide carrier can be synthesized by
standard Fmoc-chemistry using active esters or in
situ/preactivated amino acids (e.g. TBTU/HOBt/NMM in NMP)
amino acid active esters or symmetric anhydrides (see e.g.
Atherton 1989) on a suitable solid phase which include com-
mercially available polymers as Polyhipe, Wang, Novasyn as
well as any other polymers that can be derivatized and are
insoluble in the solvents used for synthesis. Depending on
the type of solid-phase polymer, the substitution should be
between 0.01 and 0.1, preferably 0.05 to 0.1 and even more
preferably about 0.05 mequivalents pr. gram solid phase;
Novasyn is typically used at about 0.05 mequivalents pr.
gram. The commercially available Novasyn KB resin, which
contains the HMB-linker (4-hydorxy-methyl benzoic acid),
hydroxyl groups at around 0.150 mequivalents pr. gram are
available for peptide coupling. A substitution of 0.05 mequi-
valents pr. gram is then achieved by performing the
esterification of the C-terminal amino acid residue of the
peptide with a short incubation time, resulting in partial
substitution only of the hydroxyl groups of the linker.
Subsequently, and before alpha-amino deprotection of the
coupled amino acid, additional free hydroxyl groups are
blocked by incubation with a large surplus of acetic
anhydride in the presence of the estrification catalyst N,N-
dimethylaminopyridine (DMAP). After peptide synthesis, HMB
can be cleaved to form either the free acid, the carboxamide,
the acylhydrazine, etc. The linkage of the peptide to the
solid-phase can be, but is not limited to be, constituted by
base-cleavable linkers. Any other scheme employing orthogonal
or graded protection of side-chains, protection of α-amino-
groups and stability of the linker is amenable. In a prefer-
able method lysine residues are orthogonally protected in
their epsilon-amino groups as e.g. Fmoc-K(Mtt)-OH, Fmoc-
K(Dde)-OH and Fmoc-K(Aloc)-OH, in which side chain protecting
groups are removable by 1% TFA, 2% hydrazine and Pd(0)(catal-
ytic hydrogenation), respectively. After synthesis, alpha-
amino lipid-coupling and selective lysine-side chain demask-
ing, synthesis or coupling of branch peptides or moieties can

then be accomplished with no risk of interference from
unmasked functional groups in the side chains of other amino
acids in the backbone peptide, especially the carboxylic acid
deriving from E. If used unprotected, this side chain carbox-
ylic group may lead to formation of lactams with any free
amino group generated during branch peptide synthesis or
coupling or with the amino groups of the backbone lysine
residues themselves, due to the generation of or presence of
carboxylic activating species during branch peptide synthesis
or coupling.

Also preferred is a backbone peptide carrier containing two
types of protection for lysine, a selectivily cleavable one
for attachment point lysines as well as Boc-protected lysines
at the "c"-non-attachment positions.

Especially preferred is a solid-phase backbone peptide com-
plex, in which a selectively protected subgroup of lysine
residues constitute attachment points after deprotection, and
in which all other protection groups as well as the solid-
phase linker are cleaved by the same chemical treatment,
preferably 95% TFA with appropiate scavengers.

Other preferred embodiments of this type include Boc-chemi-
stry with benzyl ester side-chain protected amino acids, the
peptide being linked to the solid phase through base-labile
linkers such as HMB, 3-nitro-4-(2-hydroxyethyl)benzoic acid
(NPE), 4-nitrobenzophenone oxime or photo-cleavable ones like
the acid-stable 2-bromopropionyl-α methylphenacyl ester,
Fmoc-chemistry (tBu and Boc side-chain protection) in combin-
ation with typical Boc-linkers (cleavable by HF or TFMSA
only) such as substituted benzyl esters (chloromethylphenyl
or 4-hydroxymethylphenylacetic acid (PAM)), benzhydrylamine
derivatives (e.g. 4-methylbenzhydrylamine) leading to peptide
carboxamides on HF-cleavage or photocleavables like the α-
bromo-phenacyl type mentioned above, 3-nitro-4-hydroxymethyl-
benzoic acid (ONb), and 3-nitro-4-aminomethylbenzoic acid
(Nonb, leading to the peptide carboxamide). Both strategies

may also be used with hydroxy-crotonyl type linkers cleavable by catalytic hydrogenation by Pd(0) in the presence of a weak nucleophile (see e.g. Atherton 1989). A group of particularly useful linkers are water-cleavable moieties such as glycolic acid derivatives (Hoffmann 1994).

In one embodiment, the present invention comprises a non-dendritic lipopeptide carrier ("backbone"), preferably consisting of between 10 and 50 amino acids with an α-amino-bound fatty acid, preferably palmitic acid or myristic acid or tripalmitate-Cys (see Fig. 4C), and covalently bound by its C-terminal carboxylic function to a suitable solid phase by a specifically cleavable linker.

This type of peptide can preferably be synthesized on the base-cleavable HMB-linker coupled to the solid phase. Acid-labile side-chain protection is used, for lysine (K), Boc (tert-butoxycarbonyl-). After synthesis of the protected solid-phase bound peptide chain, the amino-terminal Fmoc-group is removed by treatment with piperidine, and palmitic acid is coupled as the symmetric anhydride or by TBTU/-HOBt/NMM in NMP until the Kaiser test is negative. Subsequently, lysines are side-chain-deprotected by TFA/water (95%, 1 hour, room temperature) or by TFA/DCM (50%, 6 times 30 minutes, room temperature) and washed extensively before further use or drying and storage. The optimal loading density of the peptide on the solid phase is typically lower than the initial density of functional groups on the solid phase. The loading density is controlled in the esterification step (coupling of the first amino acid to the solid phase) by varying the incubation time; a short incubation time gives a lower loading density. It was found that loading densities in the range 0.05 to 0.1 milliequivalents pr gram solid phase was optimal when using Novasyn resins.

After attaching branch-moieties, the complex can be liberated as a free acid by cleavage with aqueous NaOH. By cleavage with methanolic ammonia, a peptide amide is liberated, the C-

terminal amide being more advantageous with regard to sup-
porting α-helical structures. Cleavage with hydrazine in DMF
or NMP leads to the peptide hydrazide.

The lipopeptide backbone contains a number of freely access-
ible functional groups, preferably amino groups, preferably
more than 2, spaced evenly along the peptide-chain. In a
variation of this structure, the lipopeptide backbone conta-
ins as functional groups carboxylates. In yet another vari-
ation, thiols are included as the functional groups. In still
another variation carbonyls, haloacetyls, hydrazides, α-
oxoacyl, amino oxyacetyl (i.e. hydroxylamine), cysteine,
maleimide-groups are derived by chemical methods from primary
amino groups. Antigenic entities, or other molecules may be
coupled stepwise or en bloc to these backbone-peptide groups
and may be peptides/ carbohydrates/haptens.

Peptides are readily amenable to stepwise chemical synthesis
on primary side-chain localised amino-groups, preferably the
ε-amino group of lysine or the delta-amino group of
ornithine. These groups also can function as targets for the
chemoselective ligation (see below) of unprotected peptides,
carbohydrates, or any other entity containing a suitable
functional group. En bloc coupling may be performed by chem-
ical methods well-known to those skilled in the art of conju-
gating proteins to proteins and peptides to proteins, includ-
ing methods for temporary protection of side-chain
functionalities in the molecule to be coupled. Such preferred
coupling methods include: coupling with glutaric aldehyde
(coupling to amino- and thiol groups), carbodiimides (coupl-
ing to amino groups), m-maleimid benzoyl-N-hydroxysuccinimide
ester (coupling from amino- to thiol-groups) carbohydrates
through oxidation followed by reaction with primary amino
groups and reduction, thiols by the N-succinimidyl 3-(2-
pyridyldithio) propionate- (SPDP-) method (see e.g. van
Regenmortel 1988).

Preferred temporary protection methods include citraconyl-
ation (primary amino group protection, the protecting group
being released by low pH) and Fmoc-derivatisation by Fmoc-
succinimide (primary amino group protection, the protecting
group being released by piperidine). Also side-chain blocked
synthetic peptides may be coupled by such methods in addition
to coupling by carboxyl-activation in organic solvents.

A preferred group of methods for en bloc couplings is "chemo-
selective ligation" (reviewed by Tam (1995)) because these
methods can be used for coupling of unprotected fragments,
especially peptides, and especially synthetic peptides and
furthermore allows the coupling-polarity or -direction to be
controlled. These methods have been used to couple peptides
to peptide scaffolds including MAPs (e.g. Lu 1991) and TASPs
(e.g. WO 95/04543).

A typical example of a preferred chemoselective method is the
reaction of a thiol nucleophile with an appropiate
electrophilic group. One such reaction is the reaction of
haloacetyl groups, preferably chloroacetyl and even more
preferably bromoacetyl groups with alkylthiols leading to the
ready formation of a very stable thioether bond (Wetzel 1990,
Robey 1992). Alkylthiol groups may be introduced anywhere in
a peptide or another molecule by coupling cysteine, and
haloacetylation is readily performed on primary amino groups
by reaction with haloacetic acid anhydride (e.g. Robey 1992).
It can also be introduced during peptide synthesis using a
preformed derivative with the haloacetyl already incorporated
as the side-chain (Ivanov 1995). In a preferred embodiment,
haloacetyls are introduced on the free ϵ-amino groups on the
lipopeptide backbone peptide bound to the solid phase and the
peptide or molecule to be attached is modified with cystein
at the desired position (e.g. Lu 1991 and Tam 1993). A sim-
ilar and also preferred reaction is obtained by reacting an
acylthiol (thiocarboxylate) group with a haloacetyl group, by
which a thioester is formed (Schnölzer 1992). Acylthiol
groups can be formed at the C-terminus of the peptide to be

coupled (by commonly known methods of solid-phase peptide
synthesis (Yamashiro 1988)); by this method, no cysteines
need to be introduced. The thioester is unstable at neutral
and basic pH-values (Tam 1995). If, however, the reaction is
allowed to take place between a N-terminal cysteine and the
acylthiol-group, a spontaneous rearrangement leads to a
stable amide bond and reestablishment of the cysteine side-
chain thiol (Dawson 1994). This thiol group, if undesirable,
can be blocked (alkylated) by methods known in the art. Other
selective reactions with thiol groups include reaction with
maleimide groups (addition reaction), leading to a stable
thioether. Maleimide groups may be introduced by acylating
primary amino groups or by coupling as the N-terminal entity
during solid-phase peptide synthesis (see Tam 1995).

Also thiol-disulfide exchange reactions leading to the
establishment of a new disulfide may be preferred as in the
SPDP-coupling scheme (Carlsson 1978). This binding although
covalent is, of course, very sensitive to reducing condi-
tions, which can be used for the deliberate release of the
molecule. Another preferred example of a chemoselective
reaction is the reaction between an aldehyde and a weak base,
preferably the reaction of aldehyde with hydrazide (acyl-
hydrazine) leading to a relatively stable hydrazone which may
be reduced to a stable substituted hydrazine (see e.g. Tam
1995) or the reaction of an aldehyde with hydroxylamine
leading to a stable oxime (Rose 1994). These reactions can be
used for the selective coupling of reducing carbohydrates or
mildly oxidized carbohydrates (monomers, dimers, oligomers,
polymers or as part of glycoconjugates oxidised by periodate)
through the aldehyde group of the carbohydrate. The reaction
of hydroxylamine or hydrazide with periodate-oxidized N-
terminal serine, threonine or cysteine (which are particular-
ly sensitive to this oxidation leading to the selective
introduction of α-oxo-acyl groups at the N-terminus in a
peptide), the preferred amino acid being serine, leads to the
chemoselective coupling of a hydroxylamine or hydrazide-
containing peptide with the N-terminal of another peptide

(Rose 1994, Gaertner 1992). Acyl-substituted hydrazines
(hydrazides) can be introduced in solid-phase peptides bound
by the HMB-linker to the solid phase by cleaving the linking
ester by hydrazine. Also, acylhydrazine may be introduced, by
derivatising a free primary amino group with Boc-monohydra-
zide succinic acid or with 4-Boc-monohydrazinobenzoic acid.
Hydroxylamine is conveniently introduced by reaction with
protected aminooxyacetic acid (see Tam 1995). In another
method, carbonyl groups are introduced on primary amino
groups by protected acetal alkanoic acids (unstable to HF and
thus limited to Fmoc-strategies of solid phase peptide syn-
thesis) (see Tam 1995). A special and preferred method
entails the reaction between an aldehyde and a N-terminal
cysteine to yield a thiazolidine. An especially useful aspect
of this method is that it introduces a heterocyclic ring that
may provide conformational stability ("rigidity") to the
peptide coupled.

From the above-mentioned examples of coupling chemistries it
is clear that, with the present invention, antigens may be
coupled through a big number of functional groups and that
antigens can be coupled in any orientation desired to the
non-dendritic backbone peptide; also, it is clear that syn-
thetic (protected or unprotected) as well as naturally-
derived peptides can be coupled, as well as other antigens,
especially carbohydrates.

The lipopeptide non-dendritic backbone as well as the com-
plete branched complex of the present invention are designed
to fold into a highly ordered structure in aqueous environ-
ment. In an especially preferred and claimed embodiment
studied in Example 1, the amino acid sequence of the oligo-
peptide is defined by a number of repeated "heptads" con-
ferring a tendency to form amphipathic parallel α-helices
(homodimeric coiled coils) to the oligopeptide. This was
based on the following design considerations:

As facing residues (a and d positions) I, L, and V are pre-
ferred. As top and bottom residues (e and g positions) D and
E and K, R, and H, respectively, are preferred. Outside (non-
interacting) residues (positions b, c, and f) are avialable
for other interactions including e.g. lactam-bridge form-
ation, histidine chelation, and attachment points.
A preference for parallel assembly is generally seen, and
when a strong hydrophobic interaction is allowed (e.g. I-L),
homodimerization is preferred, even when like-charged e and g
pairs are present, but oppositely charged e-g-pairs favours
homodimer formation.

Dimeric coiled coils are concentration dependent which is not
suitable for in vivo use. An object of the present invention
is to exclude or decrease concentration dependence by introd-
ucing further stabilising elements, typically lipidic moi-
eties.

The basic heptad was chosen as VAKLEAK. V and L constitute
big hydrophobics at the strand-to-strand interacting a and d
positions. E and K supply opposite charges at e and g pos-
itions, favouring parallel packing. The very high helix pro-
pensity residue A is occupying the rest of the positions,
except c, which is used for K creating a side-chain attach-
ment point on the outside of the helix.

The basic sequence was modified to the applied sequence:
     abcdefgabcdefg
Palm-AVHKLEHKVAKLEAKGKGKY,Palm" signifying α-N-attached
palmitic acid.

The peptide has the minimal length required for stable helix-
formation (2 turns). HX_3H is introduced at the helix-outside
vis-à-vis each other at two separate turns to create a helix-
indicating and -stabilising metal-chelating site. The N-
terminal charge is blocked by the α-N-attached palmitic acid
and the C-terminal charge (that is also counteracting the α-
helix macrodipole) is preferably blocked, e.g. as the amide.

A C-terminal GKGKY-sequence is added, however, that serves as
an α-helix C-terminal capping sequence blocking the charge.
Furthermore, this non-structured stretch can be coupled
through its C-terminal residue to other groups without af-
fecting helix-stability. Y is included as a 280 nm absorbing
reporter group and K-residues as attachment groups.

In another preferred design, the K-residues at the g-pos-
itions are selectively protected, in order to preserve the
stabilizing positive charge at the g-positions after the
deprotection subsequent to attachment of side-chain branches
to the other K-residues.

In yet another preferred embodiment, b-positions are occupied
by K-residues too, to allow for even more attachment points
on the outside of the helix.

In yet another preferred embodiment, a lactam-bridge, stabil-
izing the helix is formed between outside-residues at subse-
quent b and f positions in one of the heptads, preferably the
N-terminal one, by substituting the H-residues with a E/K-
pair. This is accomplished easily by a skilled operator by
employing orthogonal protection of these side-chains and
affecting lactam-formation by BOP/DIEA or TBTU/HOBt/NMM or
analoguous activation methods in situ with the peptide still
attached to the solid phase.

The structure of this complex leads to a high-density pres-
entation of attached antigenic entities to the surroundings,
either by interaction with hydrophobic surfaces by the
lipidic part of the structure or further enhanced by self-
aggregation into micellar structures, in both cases retaining
the dimer-structure, even at low concentration.

Moreover, the close packing of attached peptidic antigens
allows neighbour-strand interactions that support inherent
conformational traits in the peptides, thereby increasing the
similarity of the peptide conformation with the conformation

of the same peptide as part of a polypeptide sequence, a
major concern in the art of mimicking natural epitopes by
short synthetic peptides. Additional peptides ("auxiliary"
peptides) may be introduced, e.g. T-cell stimulating pep-
tides.

The structure thus represents a totally synthetic, fully
chemically defined immunogen. The peptide carrier part (the
lipopeptide backbone) itself is not immunogenic when derivat-
ized. It is characteristic of the lipopeptide backbone that
it can be synthesized by standard solid-phase chemical
methods and that antigens, peptides, carbohydrates or haptens
and naturally-derived or synthetic molecules may be coupled
to the premade solid-phase bound lipopeptide by known methods
and that peptides may be synthesized directly on the solid-
phase-bound structure by known solid-phase peptide synthesis
methods. Following liberation from the solid phase the whole
complex form stable aggregates that can be analysed by HPLC
and used for immunization. Attached peptides are sequencable
without interference from the oligopeptide lipopeptide. When
formulated together with Iscom-forming substances, the com-
plex is inserted into the Iscom-membrane by its lipid part,
presenting the branched structures on the outside.

In a specific variant of the solid-phase chemistry, the
simultaneous synthesis, but separate release of both peptide-
polymer-bound as well as free peptide can be accomplished. It
is often useful to be able to test the reactivity of the
antibodies obtained after immunization with the free peptide.
The lipopeptide-structure in another preferred embodiment is
designed to present a free amino-terminus. This is done by
coupling the lipidic part of the molecule to a side-chain of
an internal amino acid. This lipid-coupled side-chain is
preferably situated at or near one of the termini of the
lipopeptide.

In yet another configuration, the basic structure is combined
with additional (auxiliary) peptides (T-cell epitopes, tuft-

sin or other immunomodulating substances), either as an
integral part of the lipopeptide or a part of the coupled
antigens.

In a variant of the invention, the structure is designed to
incorporate the peptidic antigen as a loop in a linear
sequence, creating loop-mimetics that will also incorporate
into lipohilic membranes.

In yet another embodiment, the structure simply functions as
an anchor attaching carbohydrates to lipohilic membranes. In
this version, the peptide is synthesized as the hydrazide,
the hydrazide being highly reactive with carbonyl groups on
gently oxidised carbohydrate, and the peptide sequence incor-
porating T-cell epitopes, tuftsin or other immunomodulating
auxiliary peptides. The peptide hydrazide is obtained by
cleaving the 4-hydroxymethylbenzoic acid linker with
hydrazine.

In yet another embodiment of the structure, a specific
"peptide nucleic acid" ("PNA") sequence or a DNA-intercalat-
ing substance may be included with the purpose of binding a
specific piece of DNA by hybridization.

The invention, in addition to the use of using such con-
structs for immunization with the aim of inducing antibodies
against the antigenic branches, also comprises using such
constructs as therapeutics as well as using such constructs
as diagnostics.

The invention also relates to diagnostic embodiments using
the peptide of the solid phase complex according to the
invention, to diagnostic compositions containing said
peptide, and to methods for the use thereof in diagnosis of
diseases and pregnancy. The invention further relates to
vaccines in which a vaccine component comprising the peptide
of the solid phase complex to which an immunogenic agent is

linked and to methods of immunizing animals and conferring
resistance against diseases using said vaccine component.

Another important aspect of the invention relates to a thera-
peutic component in which a therapeutic agent is linked to
the peptide of the solid phase complex, to therapeutic compo-
sitions containing the therapeutic component and the use use
thereof for treatment and/or prevention of diseases or for
regulations of the immune response.

In clinical diagnostic embodiments of the invention, a diag-
nostic agent linked to the peptide of the solid phase complex
forming a diagnostic component of the inventions may be used
in combination with appropriate means, such as a label, to
determine the presence of a specific molecule via its binding
to the diagnostic component. In particular, the invention
relates to the diagnosis of infectious diseases derived from
bacteria, vira, and parasites as well as detection of cancer-
ous diseases, malignant tumours, and autoimmune diseases.
Furthermore, the invention can be used in the detection of
pregnancy by linking molecules capable of binding to mol-
ecules indicative of or derived from pregnancy to the peptide
of the invention, and this aspect therefore constitutes
another interesting part of the invention.

The diagnostic agent to be linked to a peptide of the solid
phase complex of the invention may be any molecule. It may be
naturally derived or chemically synthezised. A particular
interesting aspect of the invention is the linking of a
polypeptide, a carbohydrate, a lipid, and any glycosylated or
lipidated form thereof or a nucleotide sequence which is
capable of binding to molecules derived from or indicative of
pregancy or a disease, including cancerous diseases, autoimm-
une diseases and infectious diseases.

Typical methods of detection might utilize, e.g., radioactive
species, enzyme-active or other marker ligands such as
avidin/biotin and hapten/anti-hapten detection systems, which

are detectable directly or indirectly. In preferred diagnos-
tic embodiments, one will likely desire to employ an enzyme
tag such as alkaline phosphatase or peroxidase rather than
radioactive or other reagents that may have undesirable
environmental effects. Enzyme tags, e.g., often utilize
colorimetric indicator substrates that are readily detectable
spectrophotometrically, many in the visible wavelength range.
Luminescent substrates could also be used for increased
sensitivity.

One particular interesting embodiment of the invention is the
use of an antigen as the diagnostic agent. In both immuno-
diagnostics and vaccine preparation, it is often possible and
indeed more practical to prepare antigens from segments of a
known immunogenic polypeptide or carbohydrate. Certain epito-
pic regions may be used to produce responses similar to those
produced by the entire antigenic polypeptide or carbohydrate.
Potential antigenic or immunogenic regions may be identified
by any of a number of approaches, e.g., Kyte-Doolittle
antigenicity analyses (see, e.g., Kyte and Doolittle, 1982;
or U.S. Patent No. 4,554,101). Hydrophobicity analysis
assigns average hydrophobicity values to each amino acid
residue and from these values average hydrophilicities can be
calculated and regions of greatest hydrophilicity determined.

Preferred immunoassays are contemplated as including various
types of enzyme linked immunoassays (ELISAS), immunoblot
techniques, and the like, known in the art. However, it is
readily appreciated that utility is not limited to such
assays, and useful embodiments include radioimmunoassays
(RIAs) and other nonenzyme linked antibody binding assays or
procedures. Other immunodiagnostic embodiments of the inven-
tion may be based on immunoprecipitation assays, agglutin-
ation assays or the like.

Methods of diagnosing pregnancy or disease are also included
in the invention. In one embodiment, an antibody-based method

includes obtaining a sample from a patient suspected of
having the disease or being pregnant, exposing that sample to
the diagnostic component of the invention to which one or
more epitopes of a polypeptide, carbohydrate or the like,
derived from or indicative of the disease to be diagnosed are
linked and finally determining a reactivity of the antibody
that may be in the sample with one or more such epitopes. The
substantial immunological reactivity measured is indicative
of the presence or absence of the disease. Typical samples
obtainable from a patient include human serum, plasma, whole
blood, cerebrospinal fluid, seminal or vaginal fluids, exuda-
tes, and the like.

Several variations of antigen-based methods are contemplated
for development; e.g., an indirect ELISA using one or several
epitopes attached to the peptide of the invention alone or in
various combinations as antigen(s). Optimal concentration of
the antigen could be determined by checker board titration
and diagnostic potential of the epitope or whole molecule,
such as a polypeptide or carbohydrate, from which the epitope
is derived. The assay may be further examined or improved by
testing serum from e.g. an experimental animal at different
stages of a disease. These results could indicate the rela-
tive time course for sera conversion for each of the assays.

In further aspects, the present invention concerns a kit for
the detection of a molecule, including a polypeptide, a
carbohydrate or a nucleotide sequence capable of binding to
the specific selected diagnostic agent linked to a peptide of
the invention forming the diagnostic component of the inven-
tion, together with means for detecting a specific binding
between the diagnostic component and the molecule capable of
binding thereto. Examples of suitable means include labels
attached directly to the diagnostic component, or a secondary
antibody having specificity for the diagnostic component.
Alternatively, avidin-biotin mediated *Staphylococcus aureus*
binding could be used. For example, the monoclonal antibody

may be biotinylated so as to react with avidin complexed with
an enzyme or fluorescent compound.

A particular embodiment of the invention concerns kits for
detection of antibodies directed against an antigen linked to
the peptide of the solid phase complex. The antigen for the
kit(s) may be a polypeptide, a carbohydrate, a lipid or a
part thereof or a nucletide sequence obtained from a natural
source or manufactured synthetically. Alternatively, the
antigen may by produced by a recombinant DNA vector in *E.
coli* or another bacterial or nonbacterial host. Samples for
the assays may be body fluids or other tissue samples from
humans or animals. The presence of reactive antibodies in the
samples may be demonstrated by antibody binding to the diag-
nostic component followed by detection of the antibody-
antigen complex by any of a number of methods, including
ELISA, RIA, fluorescence, agglutination or precipitation
reactions, nephelometry, or any of these assays using avidin-
biotin reactions. The degree of reactivity may be assessed by
comparison to control samples, and the degree of reactivity
used as a measure of present or past infection or disease.
The assay(s) could also be used to monitor reactivity during
the course of a disease, e.g., to determine the efficacy of
therapy.

The term "a substantial immunological reactivity" is meant to
designate a marked immunological binding between an anti-
body/antiserum on the one hand, and on the other a diagnostic
component under well-defined conditions with respect to
physicochemical parameters as well as concentrations of
diagnostic component. Thus, a substantial immunological
reactivity should be clearly distinguishable from a non-
specific interaction between an antibody/antiserum and a
diagnostic component. This distinction can, for instance, be
made by reacting the antibody/antiserum with a known concen-
tration of a diagnostic component which has previously been
shown not to react with the antibody/antiserum, and then
using this reaction as a negative control. A positive control

could suitably be the reaction between the antibody/antiserum
and the same concentration of the diagnostic component used
for the immunisation resulting in the production of the
antibody/antiserum.

By the term "epitope" is meant the spatial part of an antigen
responsible for the specific binding to the antigen-binding
part of an antibody or of a T-lymphocyte.

By the term "polypeptide" is understood a molecule comprising
at least two amino acids joined by a peptide bond. The term
polypeptide thus indicates small peptides (less than 10 amino
acid residues), oligopeptides (between 10 and 100 amino acid
residues), proteins (the functional entity including at least
one peptide and/or prosthetic groups and/or glycosylation
and/or lipidation, such as lipopolypeptides and glycopolypep-
tides, etc.) as well as traditional polypeptides (more than
100 amino acid residues). Interesting polypeptides for link-
age to the branched polymer of the invention are recombinant
polypeptides which may be prepared in accordance with methods
generally known.

Particularly interesting molecules which may be used alone or
in combination with the linking of other molecules to the
peptide of the invention and the linking of which therefore
constitute an important aspect of the invention are mediators
or immunomodulators such as cytokines or bioactive cytokine
sequences which have important functions in the regulation of
the immune response, in particular T-cell reactions.

A particularly interesting aspect is the use of recombinant,
synthetically prepared, or native mediators, e.g cytokines or
a part thereof having cytokine activity inserted into
immunogenic complexes such as Iscoms or other carriers such
as microparticles together with a peptide of the invention to
which at least one other molecule is linked. Other combina-
tions may be immunogenic complexes carrying cytokines or a
part thereof having cytokine activity in mixtures with

immunogenic complexes carrying branched peptide-construct or
it may be immunogenic complexes carrying peptides of the
invention to which an active part of an immunomodulator is
linked in combination with other peptides. Among the cyto-
kines which may be of relevance are interleukin 1 - 18, TNF
(tumour necrosis factor), lymphotoxin, and interferon-alpha,
-beta, -gamma. Alternatively, bioactive cytokine-specific
sequences having cytokine activity may be part of the
sequence linked to the peptide of the invention.

A vaccine composition prepared using a vaccine component in
which an immunogenic agent is attached to the peptide of the
solid phase complex of the invention is also part of the
invention, the amount of the vaccine component being effec-
tive to confer substantially increased resistance to the
infection in question in an animal, including a human being,
against an infectious agent such as a bacteria, a virus or a
parasite as compared the degree of resistance present in an
animal not previously exposed to the infectious organism.
Thus, the invention also relates to the use of an immunogenic
component for preparing a vaccine composition and to a method
for immunizing an animal, including a human being, against an
infectious organism using the vaccine composition of the
invention.

Suitable immunogenic agents include polypeptides, carbohy-
drates, lipids or nucleotide sequences alone or in various
combinations, optionally together with other substances, such
as an immunomodulator mentioned above or a chemical compound
capable of increasing the immunogenic effect to the vaccine
component. Interesting aspects of the invention include
various combinations of immunogenic agents derived from
different infectious organisms whereby a vaccine composition
capable of conferring increased resistance to several infec-
tious organisms is obtained.

The vaccine composition may optionally be formulated in
combination with a pharmaceutically acceptable carrier or

vehicle and the vaccine optionally further comprising an
adjuvant.

By the term "conferring substantially increased resistance to
infections" is meant that the administration of the vaccine
composition to the animal has the effect that disease caused
by infections with an infectious agent is avoided or dimin-
ished or at least that the risk of catching the disease is
significantly reduced.

Typically, the vaccine composition according to the invention
are prepared as injectables either as liquid solutions or
suspensions; solid forms suitable for solution in, or suspen-
sion in, liquid prior to injection may also be prepared. The
preparation may also be emulsified. The vaccine composition
is often mixed with excipients which are pharmaceutically
acceptable and compatible with the active ingredient. Suit-
able excipients are, for example, water, saline, dextrose,
glycerol, ethanol, or the like, and combinations thereof. In
addition, if desired, the vaccine composition may contain
minor amounts of auxiliary substances such as wetting or
emulsifying agents, pH buffering agents, or adjuvants which
enhance the effectiveness of the vaccine composition.

The vaccine compositions are conventionally administered
parenterally, by injection, for example, either subcutaneous-
ly or intramuscularly. Additional formulations which are
suitable for other modes of administration include supposi-
tories and, in some cases, oral formulations. For supposi-
tories, traditional binders and carriers may include, for
example, polyalkalene glycols or triglycerides; such supposi-
tories may be formed from mixtures containing the active
ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral
formulations include such normally employed excipients as,
for example, pharmaceutical grades of mannitol, lactose,
starch, magnesium stearate, sodium saccharine, cellulose,
magnesium carbonate, and the like. These compositions take
the form of solutions, suspensions, tablets, pills, capsules,

sustained release formulations or powders and contain 10-95% of active ingredient, preferably 25-70%.

The vaccine composition may be formulated as neutral or salt forms. Pharmaceutically acceptable salts include acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccine compositions are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgement of the practitioner. However, suitable dosage for humans are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from about 1 μg to 500 μg, especially in the range from about 10 μg to 50 μg. Suitable regimes for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like, including nasal application and application on other suitable body surfaces. The dosage of the vaccine will

depend on the route of administration and will vary according
to the size of the host.

Various methods of achieving adjuvant effect for the vaccine
composition of the invention include use of agents such as
aluminum hydroxide or phosphate (alum), commonly used as 0.05
to 0.1 percent solution in phosphate buffered saline, admix-
ture with synthetic polymers of sugars (Carbopol) used as
0.25 percent solution, aggregation of the protein in the
vaccine by heat treatment with temperatures ranging between
70° to 101°C for 30 second to 2 minute periods respectively.
Aggregation by reactivating with pepsin treated (Fab) anti-
bodies to albumin, mixture with bacterial cells such as *C.
parvum* or endotoxins or lipopolysaccharide components of
gramnegative bacteria, emulsion in physiologically acceptable
oil vehicles such as mannide mono-oleate (Aracel A) or emul-
sion with 20 percent solution of a perfluorocarbon (Fluosol-
DA) used as a block substitute may also be employed.

In many instances, it will be desirable to have multiple
administrations of the vaccine, usually not exceeding six
vaccinations, more usually not exceeding four vaccinations
and preferably two or three vaccinations. The vaccinations
will normally be at from two to twelve week intervals, more
usually from three to five week intervals. Periodic boosters
at intervals of 1-5 years, usually three years, will be
desirable to maintain levels of the antibodies. The course of
the immunization may be followed by assays for antibodies for
the antigens. The assays may be performed by labelling with
conventional labels, such as radionuclides, enzymes, fluores-
cers, and the like. These techniques are well known in the
art.

It is contemplated that the vaccines of the invention should
be effective in activating both arms of the immune system.
Thus, vaccines capable of eliciting a cell-mediated immune
reaction are also a part of the invention.

Methods of actively immunizing animals, including mammals
such as human beings against at least one infectious agent
with a vaccine according to the invention are also parts of
the invention. The methods generally consist of the adminis-
tration to the animal of an immunogenically effective amount
of the vaccines of the invention.

The invention further relates to a therapeutic component
comprising the peptide of the solid phase complex of the
invention to which at least one therapeutic or prophylatic
agent capable of treating or preventing a disease, such as an
infectious, cancerous or autoimmune disease is linked. The
therapeutic agent may be any agent capable of acting as a
prophylactic agent, a therapeutic agent, or as an agent in
the prevention or relapse of a disease or in the prevention
or disruption of pregnancy. The agent may be a polypeptide, a
glycopeptide, a lipopeptide, a phospolipid, a polysaccharide,
a lipopolysaccaride, a carbohydrate, a nucleotide sequence or
any combination or modifications thereof.

In a particular interesting embodiment of the invention, a
therapeutic component comprises one or more therapeutic or
prophylactic agents in combination with at least one
immunogenic substance or immune mediator capable of control-
ling or enhancing the effect of the therapeutic or prophylac-
tic agent. The immunogenic substance or immune mediator may
be any molecule such as a carbohydrate or a peptide or a
nucleotide which can be naturally, synthetically or recombi-
nantly derived, or the mediator may be a chemical compound.

The invention also relates to any therapeutic composition
containing a therapeutic component of the invention. Thera-
peutic compositions comprising one or more therapeutic compo-
nents to which different therapeutic agents capable of pre-
venting or treating the same or different diseases or dis-
rupting pregnancy constitute another aspect of the invention.
The invention further relates to the use of a therapeutic
composition of the invention in the prevention or treatment

of a disease, or the prevention or disruption of pregnancy.
The therapeutic composition may be administered in any con-
venient way, including intramuscularly, subcutaneously,
intradermally, orally, nasally, and intravenously.

One interesting aspect of this part of the invention consists
of a therapeutic composition comprising a therapeutic compo-
nent of the invention attached to a carrier, such as an
immunostimulating complex in combination with at least one
mediator or a part thereof having mediator activity. The
mediator or a part thereof having mediator activity may be
linked to the peptide of the invention and may be further
attached to an immunostimulating complex which may be the
same or different from the immunostimulating complex carrying
the therapeutic component. Particular interesting mediators
include immunomodulators, such as cytokines including inter-
leukins, interferons, tumour necrosis factors or a part
thereof having immunomodulator activity.

Another interesting aspect of this part of the invention
comprises a therapeutic component to which a molecule capable
of binding to a target molecule present at a specific loca-
tion in a body, including a human being, is attached thereby
directing the therapeutic component to said specific location
at which the therapeutic component is to exert its effect.
The molecule capable of binding to a target molecule provide
means for targeting the activity of the therapeutic agent.
Antibodies directed against and capable of binding to the
target molecule constitute interesting examples of such
molecules.

Another aspect of the invention relates to a pharmaceutical
composition for the prevention after establishment of being
in a high risk group of developing an autoimmune disease,
treatment or prevention of a relapse of an autoimmune dis-
ease, a cancerous disease or an infectious disease caused by
an infectious agent, said composition comprising the branched
polymer of the invention to which a therapeutic agent or a

combination of various therapeutic agents are linked. The pharmaceutical composition may be formulated according to known methods based on pharmaceutically acceptable excipients.

Furthermore, the invention relates to the use of a pharmaceutical composition according to the invention for combatting, prevention or treatment of infectious agents, autoimmune disease or cancerous diseases.

The non-dendritic peptides may be used without addition of adjuvant or in combination with adjuvants like Freund's adjuvant and aluminium hydroxid or with and without insertion into immunostimulating complexes (Iscoms) or liposomes.

For immunization purposes, any mouse strain (inbreed and outbreed), rabbits, guinea pigs, minks or other animals may be used. Both humans and animals may be vaccinated with the non-dendritic peptide. The number of and intervals between immunizations may be varied. The non-dendritic peptide carrier-immunogenic complex according to the present invention may be used for ip., sc., im., iv., oral, nasal, anal, vaginal etc. immunizations. Different amounts of immunogenic peptides may be used. In addition, the non-dendritic peptide carrier-immunogenic complex may also be used for the induction of amamnestic responses.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the present invention relates to a non-dendritic peptide carrier coupled e.g. by its C-terminus through a specifically cleavable linker to a solid phase forming a peptide carrier-solid phase complex. The non-dendritic peptide carrier preferably comprises about 10-50 amino acids capable of forming a secondary structure in a benign buffer after liberation from the solid phase, the peptide complex further comprising an immunogenic substance coupled thereon. The secondary structure in the benign buffer

results in a stable carrier function of the non-dendritic
peptide when liberated from the solid phase. The immunogenic
substance coupled to the non-dendritic peptide include
antigenic substances which is covalently coupled thereon.

The secondary structure may be any structure selected from
the group α-helices, β-strand, β-turns, γ-turns, zinc-finger
structures as well as combinations thereof. The primary
function of the structure is to secure that the immunogenic
substance is presented to the environment in a stable and
predictable form. Accordingly, any of the secondary struc-
tures which in the given circumstances provides a suitable
orientation of the immunogenic substance is within the scope
of the invention.

The amphipathic α-helix in general is a well-studied (e.g.
Mant 1993) and relatively simple peptide structure that
retains a well-defined conformation in water. An amphipathic
helix has a hydrophobic side occupying one half of the helix
along its axis and a hydrophilic side on the other half.
Amphipatic α-helices typically combine in "bundles" shielding
the hydrophobic faces and exposing the hydrophilic side to
form parallel or antiparallel homodimers (Zhu 1993) or hete-
rodimers and oligomers (Zhu 1992).

Amphipathic helices are generally characterized by "heptads"
dictated by the periodicity of the α-helical turn, in which
positions a, b, c, d, e, f, and g are occupied by amino acid
residues following some simple rules: Positions a and d are
mainly occupied by hydrophobic amino acids (especially
leucine, isoleucine, and valine, and to a lesser extent
alanine, phenylalanine, tyrosine, methionine, and tryptophan)
while other positions are free to any other residue except
proline and glycine; there may be a bias towards residues
with high helix propensities (alanine, arginine, leucine,
lysine, methionine, glutamine, glutamic acid, isoleucine).

The exact arrangement by which a pair of α-helices combines
to form an inter-molecular complex can be further restricted
by additional design rules (see e.g. Mant 1993 and Zhu 1993),
e.g. by selecting a/d residues of varying hydrophobicity and
size (alternating "small" and "big" (e.g. alanine and leucine
respectively) destabilises parallel packing and stabilises
anti-parallel packing) and by either choosing e and g posi-
tions of opposite charges (favouring a parallel orientation)
or of the same charge (favouring an anti-parallel orienta-
tion) (see e.g. WO95/31480 and Chang 1994).

It is evident that this kind of structure is particularly
robust and versatile with a attractive degeneracy increasing
the freedom of design (see e.g. the "semirandom" design by
Kamtekar (1993). The peptide lenght required to form a helix
is in the practical range of chemical synthesis (minimal
lenght is around 15-20 amino acids (Kamtekar 1993, Bryson
1995, Fezoui 1995)). Metal ions chelated by H-XXX-H histidine
pairs in an α-helix (see e.g. Regan 1995) can be used to
probe and stabilize α-helical structures.

Secondary structures can further be stabilised by metal-ion-
chelation as well as by lactam- or disulfide bridges.

Non-dendritic peptide carriers according to the invention may
also include mixtures of two or more different secondary
structures. Examples of such mixed structures will often
include α-helices, e.g two α-helices joined by a β-turn. In
addition, the non-dendritic peptide may in part include a
peptide with no well defined secondary structure selected
from the group of random coil, Ω-loop and undefined loop or
combinations thereof. The peptide or peptide fragment having
no well defined structure has no influence on the non-
dendritic peptide carrier's capability of forming a secondary
structure in a benign buffer. The number of amino acids in
the part having no secondary structure contributes to the
overall number om amino acids of the non-dendritic peptide
carrier according to the present invention.

A specific and well-studied metal-ion stabilized, composite
structure is "zinc-finger" domains characteristic of DNA-
binding proteins, having the general structure Y,F-X-C-$X_{2,4}$-
C-X_3-F-X_3-H-$X_{3,4}$-H, where X can be any amino acid (Krizek
1991) and where Zn^{++} is tetrahedrally coordinated by the C
and H residues of two different strands. The resulting Zn-
supported ßßα-structure is stable in water. A 26 amino acid
residues consensus-sequence has been defined by Krizek (1991-
) (PYKCPECGKSFSQKSDLVKHQRTHTG) and used by Bianchi (1995) for
creating combinatorial libraries exploiting the stability of
the α-helix to substitutions along the peptide chain. Stru-
thers (1996) designed a 23 amino acid-residue conformational-
ly stable structure based on the Zn-finger sequence but not
needing Zn-support.

In one embodiment, the peptide carrier is in the form of a
non-dendritic peptide which forms an amphipathic helix in a
benign aqueous solution as the result of an intramolecular
anti-parallel arrangement of two α-helices joined by a turn
in a monomer super-secondary hairpin structure.

It is preferred that the non-dendritic peptide is stabilised
by the chelation of divalent metal-ions selected from Cu++,
Co++, Zn++, Ca++, Ni++, and Cd++.

In general, the secondary structure of the non-dendritic
peptide carrier is obtained by the result of the inclusion in
the peptide of one or more α-helical -, β-strand-, turn- or
zinc-finger inducing sequences of amino acids, respectively,
or combinations thereof.

In one embodiment, the secondary structure may be induced by
structure-nucleating molecular building blocks selected from

i) the α-helix inducers aminoisobutyric acid, acetylpropyl-
prolin with a thiamethylen bridge and HX_3H, any of the H-
residues being replaceable by C, chelating a divalent metal
ion; and

ii) the β-strand inducers 4-(2-aminoethyl)6-dibenzofuran and
diacylaminoepindolidione and HXH, any of the H residues being
replaceable by C, chelating a divalent metal ion; and
iii) the β-turn-inducers (S)-α-methylproline and HX$_2$H, any of
the H residues being replaceable by C, chelating a divalent
metal ion; and

iv) generally stabilizing molecular building blocks as
proline and thiazolidine.

In a further embodiment, the peptide is linked by its C-
terminus to a dibranching molecule presenting two molecules
of the peptide in parallel. The dibranching molecule may be
known in the art such as ornithine and lysine.

In a preferred embodiment, the non-dendritic peptide carrier
according to the invention gives rise to parallel homodimers
of the α-helix coiled-coil type.

For the attachment of the immunogenic substance, the non-
dendritic peptide carrier according to the invention in one
embodiment carries at least 2 attachment points, generally
defined as derivatizable and accessible functional groups.
These functional groups include hydroxylamine-, amino-,
hydrozy-, thiol-, haloacetyl-, carbonyl-, α-oxoacyl, 1,2-
thiolamin-, acylhydrazine, alkylthiol-, acylthiol, carboxyla-
te-groups, and mixtures thereof. The number of attachment
points is selected in accordance with the desired number of
immunogenic substances of the final product, accordingly, the
non-dendritic peptide may comprise at least 4 attachment
points or any other desired number which is possible accord-
ing to the lenght of the peptide.

The attachment points may be functional groups well known in
the art. Preferred attachment points are chosen from the
group of ε-amino groups or derivatized ε-amino groups in the
side-chain of lysine and a free α-amino group. In one aspect,
the non-dendritic peptide carrier according to the invention

comprises as attachment points, ε- and/or α-amino groups of a
lysine residue coupled to the ε-amino group of the lysine
residue of the original attachment point, thereby leading to
a double-functional attachment point, or, by further derivat-
ization with lysine residues to a multiple-functional attach-
ment point. One of the functional groups of the double-func-
tional attachment points, or at least one of the functional
groups of the multiple-functional attachment points, may be
blocked in one aspect. The blocking may be by a protecting
group cleavable by a chemical treatment such as orthogonal to
both Fmoc- and Boc-cleaving chemical treatments. The protect-
ing groups include Dde (1-(4,4-dimethyl-2,6-dioxocyclohexyli-
dine)-ethyl-) and allylic protecting groups such as Aloc
(allyloxycarbonyl)

The advantage of the procedure described above is the intro-
duction of a so-called "orthogonal" strategy for α-amino
protection and side-chain functionality-protection and solid-
phase linkage, respectively. "Orthogonal chemistries" means
that one type of protecting group is stable to the chemical
treatment used for the cleavage of the other and vice versa.
One such very popular strategy employs base-labile fluorenyl-
methoxycarbonyl (Fmoc) as an α-amino-protecting group and
acid-labile protecting groups for side-chains and acid-labile
"linkers". A range of linkers with graded labilities towards,
e.g., acids, bases, water, etc., are known, leading to the
liberation of the peptide either as a free acid or as a
specific derivative, most typically an amide.

The non-dendritic peptide carrier according to the invention
may also comprise two or more amino groups located in a side-
chain attached to the C-terminal amino acid.

In a further embodiment, a non-dendritic peptide carrier
according to the invention may comprise a subclass of ε-amino
groups in the side-chain of other lysine residues and a free
α-amino group, said subclass comprising at least one such
amino group being protected by a protecting group cleavable

by a chemical treatment orthogonal to both Fmoc- and Boc-cleaving chemical treatments.

In one aspect, the amino groups serving as attachment points may further be derivatized by chemical means selected from

i) treatment with di-acid anhydrides, preferably succinic anhydride to yield free side-chain carboxylic acids, and

ii) treatment with haloacetic acid anhydride, preferably bromoacetic acid anhydride to yield free haloacetyl groups, preferably bromoacetyl groups, and

iii) treatment with activated maleimide, preferably m-maleimide benzoyl-N-hydroxysuccinimide ester to yield free maleimide groups, or treatment with SPDD to yield free pyridyl-dithio grops, and

iv) treatment with carboxy-activated α-amino-protected cysteine to yield free thiol groups, and

v) treatment with carboxy-activated α-amino-protected cysteine to yield free thiol groups, followed by deprotection of the α-amino group to yield free 1,2 thiol-amino groups, and

vi) treatment with glutamic or aspartic acid to yield free side-chain carboxylic groups, and

vii) treatment with amino-oxyacetic acid to yield free hydroxylamine groups, and

viii) treatment with α-amino-protected, carboxy-activated serine to yield, after α-amino deprotection free 1,2-aminoalcohols, which in turn can be oxidised to free α-oxoacyl groups, and

ix) treatment with Boc-monohydrazide succinic acid to yield
free acyl-substituted hydrazines.

The non-dendritic peptide carrier according to the invention
may also comprise side-chain functional groups serving as
attachment points which are derivatized with secondary struc-
ture supporting, derivatizable building blocks, selected from

i) the α-helix inducers aminoisobutyric acid, acetylpropyl-
prolin with a thiamethylen bridge and HX_3H, any of the H-
residues being replaceable by C, chelating a divalent metal
ion; and

ii) the β-turn-inducers (S)-α-methylproline and HX_2H, any of
the H residues being replacable by C, chelating a divalent
metal ion; and

iii) the β-strand inducers 4-(2-aminoethyl)6-dibenzofuran and
diacylaminoepindolidione and HXH, any of the H residues being
replacable by C, chelating a divalent metal ion; and
iv) generally stabilizing molecular building blocks as
proline and thiazolidine.

The side-chain functional groups of the non-dendritic peptide
carrier serving as attachment points may, in one embodiment,
be derivatised with a dibranching residue being selected from
the structures disclosed in Fig. 4A and Fig. 4B.

The non-dendritic peptide may, in one aspect, comprise at
least two free carboxylic acids or amino groups located in a
side-chain attached to the C-terminal amino acid, preferably
a lysine, or said side-chain attached near the C-terminal
amino acid, the said non-dendritic peptide further comprising
other attachment points not constituted by carboxylic acids.
One example of a method for preparing a non-dendritic peptide
comprising at least two carboxylic groups in a side chain
attached to or near the C-terminal amino acid comprises
introducing one or multiple glutamic acid residues or

aspartic acid residues preferably by synthesis on the ε-amino group of one selected C-terminally located lysine residue using orthogonal protection for the α-amino group, compared to the protection use for the amino acids incorporated in the rest of the peptide and demasking the side-chain carboxylic groups before proceeding with synthesis of the backbone peptide.

Similarly, a method for preparing a non-dendritic peptide comprising at least two amino groups in a side-chain attached to or near the C-terminal amino acid comprises introducing one or multiple lysine residues preferably by synthesis on the ε-amino group of one selected C-terminally located lysine residue using orthogonal protection for the α-amino group, compared to the protection use for the amino acids incorporated in the rest of the peptide and not demasking the side-chain carboxylic groups before proceeding with synthesis of the backbone peptide.

To further ameliorate the immunogenicity and versatility of the immunogenic response, various lipids with adjuvant effects may be included in the immunogenic compounds according to the invention. The basic design according to the invention has been developed to adapt a number of different antigens and other entities such as alkyl chains in the structure.

Accordingly, in a further aspect, the non-dendritic peptide carrier may comprise one or more alkyl-chains, preferably in the form of a saturated fatty acid bound covalently to the N-terminus of the peptide or to an amino acid side-chain. The carbon chain of the alkyl-chain mentioned above may comprise about 4-25 carbon atoms such as 6-20 carbon atoms, preferably 7-17 carbon atoms in length. In a preferred aspect, the carbon chain is a lipidic moiety comprising palmitic acid or myristic acid or mixtures thereof. An example of such lipid moiety is the immunostimulatory $palm_3$-Cys-molecule as shown in Fig. 4C.

In one interesting embodiment, a non-dendritic peptide car-
rier according to the invention comprises at least one
lipidic moiety bound as a thioester to the peptide, preferab-
ly through a cysteine side chain thiol group.

In an aspect the lipidic moiety is provided by fatty acids,
typically palmitic acid or myristic acid coupled to the side
chains of amino acids selected from lysine and serine. The
lysine and serine may constitute a linear peptidic chain and
preferably being of alternating chirality, a D-amino acid
being followed by a L-amino acid and a L-amino acid being
followed by a D-amino acid.

In a further embodiment, the non-dendritic peptide carrier
comprises a lipidic moiety such as palmitic or myristic acid
located at the C-terminus or near the C-terminus. In one
aspect, it is preferable that the peptide comprises no free
and accessible side-chain functionalities but an unprotected
N-terminus α-amino group.

An aspect of a non-dendritic peptide carrier according to the
invention is a carrier comprising 20-50 amino acids and which
comprises in its sequence repeated "heptads" having the form
"abcdefg". Preferably, the positions "a" and "d" are occupied
by hydrophobic amino acids selected from I, V, L, F, and A,
positions "e" and "g" by charged amino acids of opposite
signs, selected from E, D, and K. Positions "b", "c", and "f"
are preferably occupied by any amino acid residue selected
from the group of A, R, N, D, E, C, Q, E, H, I, L, K, M, F,
S, T, W, Y or V but preferably A, S, T, C, H or K, yielding
2-10, preferably 4-6 evenly spaced lysine residues with
freely accessible derivatizable ϵ-amino groups. In a pre-
ferred embodiment, at least one pair of histidines or a
histidine and a cysteine are occupying consecutive "b" and
"f" positions in the peptide chain.

In one embodiment, the repeated "heptads" are located in its
inner sequence, i.e. the sequence located within the segment

of the peptide corresponding to the segment from amino acid
2-5 to n-(2-5), preferably to the segment from amino acid 3
to n-3, where n is the number of amino acids in the peptide.
In a further aspect the lysine residues occupying "e" and "g"
positions are orthogonally side-chain protected compared to
lysine residues occupying "b", "c", and "f" positions. The
orthogonal protecting group is preferably being stable
towards treatments, such as Fmoc- as well as Boc-cleaving
treatments. In another aspect, at least one "f" position is
occupied by C.

The carrier may also comprise at least one, preferably one
lactam bridge linking a "b" and an "f" amino acid in a ter-
minally placed heptad, "b" and "f" being either E and K or K
and E, E being in both cases replaceable by D. In addition,
the carrier may comprise one Y or W included in the sequence,
preferably as one of the 2-5 C-terminal amino acids.

T-cell help is important for efficient immunizations. T-cells
are activated by interaction with peptides bound by the major
histocompatibility complex (MHC) on antigen-presenting cells.
The MHC of an individual bind a subset of peptides conforming
to the sequence definition of that individual only (see
Rammensee, 1995). "Promiscous" peptides have been described
that are bound by a large number of individual MHCs covering
a large proportion of the species in question. The art of
incorporating T-cell stimulating moieties in synthetic
peptide constructs intended for immunizations is well known.
In addition to general "stimulators" including tuftsin (-
=[Thr-Lys-Pro-Arg]$_N$) (e.g. Fridkin 1989), muramyldipeptide
(MDP = N-acetyl muramyl L-alanyl D-isoglutamine)(Ellouz
1974), and lipopeptides (Wiesmüller 1992) or simple lipids
(Flinn 1994, Vitiello 1995) a number of "promiscous" pepti-
des, binding to a large subset of MHC-molecules have been
described (Panina-Bordignon 1989). These substances can be
combined with peptide antigens in linear (e.g. WO 95/00540,
Wang 1995, Vitiello 1995 and Kaumaya 1993) or branched arran-
gements (US patent 5229490, WO 93/22343, Pawan 1994, Flinn

1994, Jackson 1995). The lipid-structure by Wiesmüller (1992)
is particularly efficient (Defoort 1992) and has even been
shown to be efficient in a linear construct with a dimeric
carbohydrate antigen (Toyokuni 1994). Importantly, a "mixed"
linear poly-epitope (recombinantly expressed), containing
only core sequences of several T-cell stimulatory peptides
has been found to stimulate all subsets binding the individ-
ual peptides (Thomson 1995). It was not shown but may be
possible to inject such DNA-constructs directly by the known
art of DNA-immunization (e.g. Ulmer 1993) and obtain a simi-
lar broad stimulation in this way.

Peptide-attachable DNA-binding substances include intercalat-
ors (e.g. quinoline (Brown 1994)) which bind DNA
unspecifically and peptide nucleic acids (PNA) which can
hybridise with DNA in a sequence-specific way (WO 95/01369).

Also, specific imunomodulators, e.g. cytokines or cytokine
fragments, may be included (e.g. Kumaratilake 1995, European
Patent 0 604 727 A1).

Accordingly, to circumvent restriction of the Major Histocom-
pability Complex, specific T-cell stimulatory peptides, in
addition to the immunogenic substance, may be included in the
immunogen complex according to the invention. This may be
done by peptide synthesis using orthogonal chemistry or by
attaching composite linear constructs.

Accordingly, a non-dendritic peptide according to the inven-
tion may comprise one or more copies of a peptide moiety or
comprising a combination of peptide moieties. The peptide
moiety may in one embodiment be situated at either or both
ends of the non-dendritic carrier in its side-chain- and N-
terminal-blocked form. Examples of such peptide moieties
include the following:

i) [TKPR]$_N$, in which N is preferably from 1-5 (tuftsin oligomer), muramyldipeptide (N-acetyl-muramyl-L-alanyl-D-isoglutamine) or variants thereof, and

ii) a T-cell stimulatory peptide selected from QYIKANSKFIGITE (tetanus toxoid 830-843) and FNNFTVSFWLHRVKVSASHLE (tetanus toxoid 947-967), DQVHFQPLPPAVVKLSDALI (Mycobacterium tuberculosis 38 kD antigen 350-369), DIEKKIAKMEKASSVFNVVNS (Plasmodium falciparum circumsporozoite protein 378-398), KLLSLIKGVIVHRLEGVE, measles virus F-protein 286-302, LDNIKGN-VGKMEDYIKKNNK (Plasmodium falciparum MSP-1, 260-279), LQTMVK-LFNRIK, NSVDDALINSTKIYSYFPSV, QYIKANSKFIGITELK, and PGINGKAIHLVNNESS; and

iii) a T-cell stimulatory peptide selected from poly T-cell-epitope constructs wherein the T-cell-epitope elements are arranged in a substantially linear construction comprising interposed minimal T-cell epitope peptide segments, preferably without flanking sequences; and

iv) a cytokine derived peptide selected from
IFN-gamma (1-39) HGTVIESLESNNYFNSSGIDVEEKSLFLDIWRNWQKDG,
IFN-gamma (95-133) AKFEVNNPQVQRQAFNELIRVVHQLLPESSLRKRKRSRC,
TNF (70-80) PSTHVLITHTI
IL-1 _beta_ (163-171) VQGEESNDK;
and combinations thereof.

In one embodiment, the non-dendritic peptide carrier may comprise the specified peptide moiety in its side-chain protected, but N-terminally unprotected form, said peptide moiety being coupled as a branch peptide to at least one side chain attachment point of a backbone peptide. An example of such attachment point is an ε-amino group in the side chain of lysine.

In a further embodiment, the non-dendritic peptide comprises the specified peptide moiety in its side-chain protected, but N-terminally unprotected form, said peptide moiety being

coupled as a branch peptide to at least one side-chain attachment point in a backbone peptide, said attachment point being a double-functional or multiple-functional attachment point. Relevant groups may be ϵ- and α-amino groups of a lysine residue coupled to the ϵ-amino group of the lysine residue of the original attachment point,

In yet another embodiment, the non-dendritic carrier comprises the specified peptide moiety in its side-chain protected and N-terminally protected or N-terminally unprotected form, said peptide moiety being coupled to at least one side-chain attachment point in a backbone peptide. The attachment point may be a double-functional or multiple-functional attachment point and in a further embodiment wherein at least one are further protected orthogonally to Fmoc and Boc and other acid-labile side-chain protecting groups.

The non-dendritic peptide according to the invention may also comprise at least one PNA moiety. The PNA may comprise a monomer sequence binding a specific DNA-molecule by hybridization. In another embodiment, the non-dendritic peptide comprises at least one functional DNA-intercalator moiety. This intercalator is in one embodiment quinoline.

In addition, the non-dendritic peptide carrier may comprise at least one DNA or RNA oligonucleotide moiety. The DNA oligonucleotide moiety is preferably one which is coupled through its 3'-end to an amino group of said peptide. Furthermore, it may comprise a nucleotide sequence encoding a T-cell stimulatory peptide. In a preferred embodiment, the oligonucleotide sequence binds a specific DNA- or RNA-molecule by hybridization.

In a preferred embodiment, the attached oligonucleotide comprises a hexanucleotide corresponding to the general formula (5')PuPuCGPyPy(3') in which Pu is a purine base, Py is a pyrimidine base and CG represents unmethylated CpG dinucleotide. This hexanucleotide motif has been shown to be

a potent inducer of cytokines in a range of immunocompetent cells (Klinman 1996).

The non-dendritic peptide carrier may comprise a spacer molecule. Preferably, the spacer is selected from G_N, where N=2-8.

The solid-phase on which the non-dendritic carrier is coupled is preferably a polymer whereby the solid phase complex represents a copolymer. It may be advantageously to bind the non-dendritic peptide to the solid phase through a linker which is selectively cleavable by a specific chemical treatment.

The solid phase is normally constituted by derivatizable polymers (matrices, resins), which are insoluble in water and in organic solvents including dimethylformamide, dimethylsulfoxide, N-methylpyrrolidone, dichloromethan, piperidine, diethylether and aqueous trifluoroacetic acid. Divinylbenzene/polystyrene and polyacrylamide polymers sometimes in combination with macroscopic support materials such as kieselguhr and derivatized with a variety of spacers and linkers. The amount of peptide on the polymer may vary according to size of the peptide or the specific conditions of the synthesis.

In one aspect, the non-dendritic peptide carrier may be substituting the solid-phase polymer to about 0.001 to about 5, preferably such as from about 0.01 to about 1, more preferred from about 0.02 to about 0.08, still more preferred from about 0.04 to about 0.06, most preferred from about 0.05 to about 0.1 mmoles pr. gram of solid phase.

The chemical treatment liberating the non-dendritic carrier peptide from the solid-phase bound linker may be orthogonal to the chemical treatment used for the cleavage of protecting groups from the intended attachment points in the side-chains of the peptide. Such linkers may be selected from 3-nitro-4-

hydroxymethyl benzoic acid type linkers cleavable by
photolysis, hydroxy-crotonyl-aminomethyl type linkers
cleavable by catalytic hydrogenation, 4-methylbenzhydrylamine
type linkers cleavable by hydrofluoric acid or trifluoro-
methanesulfonic acid, unmodified Rink-type linker (4-(2', 4'-
dimethoxyphenyl Fmoc-aminomethyl)-phenoxyacetic acid)
cleavable by aqueous trifluoroacetic acid.

In one embodiment, the non-dendritic carrier peptide is bound
as an ester to a linker to the solid-phase said peptide being
cleavable by aqueous base.

The non-dendritic carrier peptide may also be linked to a
solid-phase bound 4-hydroxymethylbenzoic acid liberating the
peptide as a carboxylic acid upon treatment with hydroxyl-
nucleophiles, as an amide upon treatment with methanolic
ammonia and as a hydrazide upon treatment with hydrazine.

The non-dendritic peptide carrier according to the invention
may, in one embodiment, be linked to the solid phase through
a linker releasing the peptide on contact with water, prefer-
ably a glycolic acid linker of the structure given in Fig. 4D
or a linker releasing the peptide through diketopiperazine
formation.

In an aspect of the invention, the non-dendritic peptide
carrier in a solid-phase complex comprises 5-50, preferably
10-20 amino acids, the peptide also comprising a covalently
bound lipidic moiety, preferably palmitic or myristic acid
which is located at the N-terminus or near the N-terminus,
the peptide being attached to the solid phase by a hydrazine-
clevable linker to form the solid phase complex.

In order to detect the non-dendritic peptide carrier accord-
ing to the invention, it may comprise a substance having
characteristic and measurable spectral or radioactive prop-
erties such as UV-absorbing properties, visibly absorbing or
fluorescent properties.

In a further embodiment, the invention is directed to the use of a non-dendritic peptide carrier as defined above as a scaffold for carrying other moieties by their covalent attachment to derivatizable groups of the said peptide. The derivatizable group is in one embodiment selected from an α-amino group, an ϵ-amino group of K, a chemically prepared derivative of an ϵ-amino group of K, and a thiol group from the side-chain of C.

In yet another embodiment, the invention is directed to the use of a non-dendritic peptide carrier as defined above as a scaffold for the production of chemical derivatives, characterised by covalently attached molecules at the attachment points. The molecules may be selected from peptides, carbohydrates, haptens, glycopeptides, lipopeptides, DNA, RNA, PNA, proteins and glycoproteins and combinations thereof.

In an additional embodiment, the invention is directed to the use of a non-dendritic peptide carrier as defined above coupled to a solid phase as a solid-phase complex scaffold for the stepwise conventional Fmoc- or Boc-based solid-phase peptide synthesis for the stepwise synthesis of peptide moieties with defined sequences on the attachment-points of the solid-phase bound peptide followed by specific cleavage of the whole complex from the solid phase.

Components which can be coupled on the non-dendritic peptide carrier according to the uses defined above may be selected from chemically synthesized protected peptides, chemically synthesized unprotected peptides, recombinant synthesized peptides, naturally-derived peptides, naturally-derived or synthetic carbohydrates, naturally-derived or synthetic glycopeptides, naturally-derived or synthetic lipopeptides, naturally-derived or synthetic nucleic acids, naturally-derived or synthetic ribonucleic acids, peptide nucleic acids, haptens or other antigens or non-antigens, or other amino-, carboxylate-, haloacetyl-, maleimide-, thiol-, 1,2-

thiol-amino-, hydroxylamine-, α-oxoacyl-, carbonyl-, and
acylhydrazine-reactive substances and mixtures thereof.

The non-dendritic peptide carriers of this invention can also
be used for the production of structured synthetic peptide
libraries which in contrast to normal peptide libraries is
characterized by a higher degree of conformational definition
achieved by a structure-supporting framework in the present
invention provided by the non-dendritic peptide carrier.
Furthermore, the libraries that can be achieved by the pres-
ent invention can be used for immunization. Thus, one par-
ticular aspect of peptide libraries derived from non-
dendritic peptide carriers according to the present invention
is that a structured library is obtained which is recognized
easily by natural antibodies. In addition, the library can be
used for immunization with the aim of achieving broad protec-
tion e.g. against a range of pathogenic virus variants.

The non-dendritic peptide carrier can be used in standard
methods of preparing peptide libraries, including the split-
combine approach which results in a population of derivatized
non-dendritic peptide carrier molecules in which only ident-
ical branch peptides are coupled to the same non-dendritic
peptide carrier molecule.

The non-dendritic peptide carrier according to the invention
may be used as a scaffold for the coupling of one or more
components selected from chemically synthesized protected
peptides, chemically synthesized unprotected peptides, recom-
binant synthesized peptides, naturally-derived peptides,
cyclic peptides, naturally-derived or synthetic carbohy-
drates, naturally-derived or synthetic glycopeptides, nat-
urally-derived or synthetic lipopeptides, naturally-derived
or synthetic nucleic acids, naturally-derived or synthetic
ribonucleic acids, peptide nucleic acids, haptens or other
antigens or non-antigens, or other amino-, carboxylate-,
haloacetyl-, maleimide-, thiol-, 1,2-thiol-amino-,
hydroxylamine-, α-oxoacyl-, carbonyl-, and acylhydrazine-

reactive substances and mixtures thereof. This use as a
scaffold can be performed when the non-dendritic peptide
carrier is liberated from the solid phase. However, it is
preferred that the coupling of one or more components as
mentioned above is performed with the non-dendritic peptide
carrier still attached to the solid phase.

The peptide carrier-solid phase complex according to the
invention may also be used as a scaffold for the attachment
of moieties through the C-terminally located carboxylic acids
in the said peptide carrier-solid phase complex, said
attached moieties being typically amine-containg compounds,
most typically synthetic or natural peptides or proteins with
which a selective coupling through their amino groups can be
achieved by known methods for conjugating peptides and pro-
teins to proteins, then cleaving off protecting groups of the
backbone lipopeptide as well as of the coupled peptides if
any, followed by liberation of the whole complex.

A further method of using the non-dendritic peptide carrier-
solid phase complex according to the invention is by combina-
ting it with a free peptide acid preferably comprising 5-20
amino acids and containing a lipidic moiety located at the N-
terminus or near the N-terminus, said lipidic moiety being
preferably palmitic or myristic acid, said peptide further
carrying no other free functional groups than the C-terminus
carboxylic acid. The method comprises coupling, preferably by
sequential peptide synthesis a target peptide to the peptide
carrier-solid phase complex, terminating the synthesis by
coupling the free lipopeptide to the α-amino group of the
solid-phase bound peptide and subsequently cleaving off
protecting groups and liberating the whole complex, which
then, upon liberation will expose the synthesized target
peptide as a loop. The peptide structure may be stabilized by
including a moiety bridging the two ends of the peptide by a
structure selected from an intramolecular lactam-linkage, an
intramolecular S-S-linkage and a suitable bifunctional spacer
molecule.

The lipopeptide hydrazide deriving from hydrazine-cleavage of
peptide carrier-solid phase complex may be used for coupling
aldehyde-containing compounds, such as reducing carbohydrates
which may be naturally-occuring or synthetic carbohydrates or
such carbohydrates mildly oxidized to accomplish ring open-
ing, said method consisting of mixing said reducing carbohy-
drate with the lipopeptide hydrazide.

Glycoconjugates chosen from glycoproteins, glycopeptides or
glycolipids, which may be glycosylated immunomodulatory
glycoconjugates selected from interleukin 6, interferon-γ,
other glycosylated cytokines and immunoglobulins may be
coupled selectively through its carbohydrate moiety to the
hydrazide group of the free lipopeptide by the above method.

A non-dendritic peptide carrier immunogen complex may be
incorporated into Immunostimulating Complexes (Iscoms) or
liposomes by virtue of the lipidic moiety resulting in a non-
dendritic peptide carrier-Iscom complex.

Two or more different complexes may be incorporated into the
same Iscom or liposome. The complexes may be used for the
preparation of a vaccine.

In a preferred embodiment, the non-dendritic peptide carrier
according to the invention is non-cyclic.

As mentioned above, the present invention relates to a method
for preparing a non-dendritic peptide carrier comprising an
immunogenic substance coupled thereon comprising the steps of

i) synthesizing a non-dendritic peptide carrier as defined in
any of the preceding claims by chemical solid-phase synthesis
on a linker, and

ii) synthesizing the immunogenic substance directly on the
non-dendritic peptide carrier, and

iii) cleaving the non-dendritic peptide carrier from the
solid phase. In addition, step ii) may further comprise
covalent attaching other moieties to the peptide carrier by
use of derivatizable groups thereon.

The non-dendritic peptide carrier according to the invention
may also be used as a scaffold for the production of chemical
derivatives, characterised by covalently attached molecules
at the attachment points, the molecules being selected from
peptides, carbohydrates, haptens, glycopeptides, lipopepti-
des, DNA, RNA, PNA, proteins and glycoproteins and combina-
tions thereof. In addition, the scaffold-peptide complex may
be incorporated into Immunostimulating Complexes (Iscoms) by
use of a lipidic moiety resulting in a non-dendritic peptide
carrier-Iscom complex. Two or more different complexes may be
incorporated into the same Iscom.

The non-dendritic peptide carrier as defined above is suit-
able as a diagnostic component for detecting a molecule or a
substance as one or more diagnostic agents, e.g an antigen or
antibody such as a polypeptide, lipopolypeptide, a glycopoly-
peptide, a phospholipid, a carbohydrate, a lipopolysaccharide
or a nucleotide sequence (including a DNA sequence, an RNA
sequence, or any modification thereof), PNA or any combina-
tions or modifications thereof which may be linked to the
carrier. In one embodiment, the diagnostic component may
comprise at least two different diagnostic agents capable of
detecting the same or different molecules.

Such a diagnostic component may be used use in vivo directly
in an animal or in vitro by use of a sample.

The diagnostic agent is used in an amount which is effective
to detectably react with said molecule to be detected and to
which the diagnostic agent is capable of binding. The diag-
nostic component is used for detection a molecule by incubat-
ing the diagnostic component with the molecule for a time
sufficient for the diagnostic component, e.g. in a suitable

composition to react with the subject and forming a complex
and detecting the presence of bound molecule by subjecting
said complex to a detecting means.

The diagnostic component be used according to the invention
for detection of a molecule derived from or indicative of
pregnancy, of a disease, such as an infectious disease, an
autoimmune disease, a cancerous disease or any other disease
wherein an indicative molecule is known, e.g by use of a
sample derived from tissue including a biopsy or tissue
extract, a cell culture or the animal, including a human
being. Accordingly, the sample may be derived from serum,
plasma, whole blood, cerebrospinal fluid, seminal or vaginal
fluids, exudates, saliva, urine, faeces, or the like.

In one embodiment, the diagnostic component is administered
directly to the aminal in which the diagnostic agent is
complementary to a molecule present in the animal and indica-
tive of the disease or pregnancy of said animal.

In a further use according to the invention, the derivatized
non-dendritic peptide carriers according to the invention is
used for the selection of specifically peptide binding oligo-
nucleotides, especially oligoribonucleotides ("aptamers").
Such aptamers have been shown to be able to bind specifically
and with high affinity to specific peptides in an induced-fit
mode resembling antibody binding (Xu 1996). Anti-peptide
aptamers are of interest as an alternative to antibodies in
diagnostic assays and in therapy based on binding to and
blocking of e.g. virus-derived mRNA-binding proteins. Ap-
tamers are selected in an *in vitro* process from a large pool
of random oligonucleotides by their binding to the solid-
phase coupled peptide. By using the derivatized non-dendritic
peptide carriers which are objects of this invention, it is
believed that better and more specifically binding aptamers
can be selected as the peptide is presented in a more well-
defined and structure-supported way. Also, in the present

invention, the selection procedure itself is simplified as
the solid-phase coupled complex is used directly.

As explained above, in a further embodiment, the present
invention relates to a vaccine component comprising a non-
dendritic peptide carrier as defined above on which at least
one immunogenic agent or mediator is attached. The
immunogenic agent may be a polypeptide, a glycopeptide, a
lipopeptide, a phospholipid, a polysaccharide, a lipopolysac-
caride, a carbohydrate, a nucleotide sequence, PNA or any
combination or modifications thereof. Furthermore, at least
one mediator capable of affecting the immunogenic effect of
the vaccine component or the reaction of the immune system
exposed to the vaccine component may also be linked or form
part of the peptide carrier.

Examples of such mediators includes a tuftsin, an immunomodu-
lator including a cytokine such as an interleukin or
interferon, an enhancer, or a part or modification of the
above having mediator activity. The mediator may be nat-
urally, synthetically or recombinantly derived.

In another embodiment, the vaccine component is attached to a
second carrier, such as an Immunostimulating Complex (Iscom)
or liposome, optionally in combination with a mediator or a
part thereof having mediator activity and attached to the
non-dendritic peptide carrier and/or second carrier.

The non-dendritic peptide carrier of this invention may also
be used with other secondary carriers, including proteinoid
microspheres constituted of derivatised alpha-amino acids
(Haas 1996) and poly(lactide-coglycolide) polymer slow
release biodegradable microparticles (PLG)(Ertl 1996).
Proteinoid microspheres are prepared by derivatized alpha-
amino acids that spontaneously form microspheres at low pH
and encapsulate antigens by simple mixing of the amino acids
with the antigen followed by acidification (Haas 1996). With
PLG, an emulsion of the PLG and the antigen is formed fol-

lowed by lyophilization (Ertl 1996). The particles preferably have diameters from 0.5 to 10 μm, preferably from 0.5 to 2 μm and in both cases are biodegradable.

Such particulate formulations have the advantage that they may be administered orally and are immunogenic by oral administration.

The invention also relates to a vaccine composition comprising at least one vaccine component as defined above. The composition preferable comprises an effective amount of the vaccine component to confer increased resistance to one or more infection(s) in the animal, the composition optionally further comprising a pharmaceutically acceptable carrier or vehicle, enhancers or adjuvants.

An example of the use of an adjuvant is the combination of the non-dendritic carrier peptide combined with Schiff-base-forming components which are known to act like adjuvants and enhance the Schiff-base formation between carbonyl-groups and amino groups on antigen-presenting cells and T-cells occuring during a natural response (Gao 1990). This can be done by including a Schiff-base forming substance in the vaccine formulation directly activating T-cells by binding to amino-groups on their surface (Rhodes 1995a) or by administering simultaneously with the immunogenic sustance the two carbohy-drate-modifying enzymes neauraminidase and galactose oxidase ("NAGO" adjuvant) (Rhodes 1995b). In a preferred embodiment, a Schiff-base forming aldehyde, as e.g. tucaresol (4(2-formyl-3-hydroxyphenoxymethyl)benzoic acid) is bound as an auxiliary moiety in the non-dendritic peptide carrier. This can be achieved by coupling to a selectively deprotected amino-group in the non-dendritic peptide carrier through activation of the carboxylic acid in tucaresol.

The vaccine composition according to the invention may be used for immunizing an animal including an human being by

administering the vaccine composition nasally, subcutaneous-
ly, intramuscularly or by any other convenient route.

In a still further aspect, the present invention relates to a
therapeutic component comprising a non-dendritic peptide
carrier as defined above to which at least one therapeutic or
prophylactic agent is attached, e.g as defined for the vac-
cine component above. A therapeutic component according to
the invention may further comprise at least one mediator, e.g
as defined for the vaccine above, capable of controlling or
enhancing the effect of the therapeutic or prophylactic agent
linked to the carrier.

As described above for the vaccine, the therapeutic component
according to the invention may also comprise a second car-
rier, such as an Immunostimulating Complex (Iscom) or
liposome, optionally in combination with a mediator or a part
thereof having mediator activity and attached to the non-
dendritic peptide carrier and/or second carrier.

In a preferred embodiment of the invention, a therapeutic
component according to the invention may also comprise a
targeting molecule capable of binding to a target substance
present at a specific location in the animal thereby direct-
ing the therapeutic component to said specific location where
the therapeutic component is to exert its effect. Such tar-
geting molecule is e.g an antibody. Accordingly, the thera-
peutic component may be capable of preventing, including
preventing relapse, or treating a disease or capable of
preventing or disrupting pregnancy in an very effective way.
Any disease wherein a target substance is identified may be
treated according to the invention. Thus, diseases to which
the invention is applicable include infectious diseases,
cancerous diseases and autoimmune diseases.

The pharmaceutical component may be used in a composition
comprising the therapeutic component together with a pharma-
ceutically acceptable carrier.

Accordingly, the present invention also relates to a method of treatment and/or prevention of a disease, comprising administering to a patient in need thereof a therapeutically or prophylactically effective amount of a pharmaceutical composition described above.

Finally, the present invention also relates to a detecting component for detecting a molecule or a substance, the component comprising a non-dendritic peptide carrier as defined above to which a detecting agent is linked.

Any range or value given herein may be extended or altered without losing the effects sought, as will be apparent to the skilled person for an understanding of the teachings herein.

DESCRIPTION OF FIGURES

Fig. 1A shows a non-dendritic peptide carrier-solid phase complex before attachment of an immunogenic substance.

Fig. 1B shows another embodiment of a non-dendritic peptide carrier-solid phase complex in which an auxiliary segment has been attached.

Fig. 1C shows a branched peptide complex consisting of a non-dendritic peptide carrier-solid phase complex on which immunogenic branch peptides have been attached.

Fig. 1D shows a similar branched peptide complex as shown in Fig. 1C which in addition comprises an auxiliary segment coupled to a double attachment point.

Fig. 1E shows a similar branched peptide complex as shown in Fig 1C which but including an auxiliary segment coupled in a linear arrangement in the branch peptide.

Fig. 2 shows HPLC-analysis of a backbone-peptide before (A) and after (B) coupling of palmitic acid (raw products)(see Example 1).

Fig. 3 shows Matrix-assisted Laser desorption time-of-flight mass spectrometry analysis of palmitoylated backbone-peptide (HPLC-purified) prepared as described in Example 1, using a Fisons linear mode mass spectrometer, and alpha-cyano 4-hydroxy cinnamic acid as the matrix. The results are calibrated and centroid adjusted.

Fig. 4A and 4B show two dibranching linkers A and B respectively.

Fig. 4C shows the structure of the tripalmitate moiety, N-palmitoyl-S-[2,3-bis (palmitoyl-oxy)-(2RS)]-propyl-[R]-cysteinyl.

Fig. 4D shows a glycolic acid linker.

Fig. 5 shows antibody reactivity of a HIV-1 seropositive donor against dilution curves of peptide, gp41 (aa598-609), being tested alone and linked, respectively, to the non-dendritic peptide carrier as described in Example 19.

Fig. 6 shows dilution curves of sera obtained from HIV-2 seropositive donors (———) and HIV-2 seronegative donors (----) tested against the non-dendritic peptide carrier construct. Positive signals were obtained at all dilutions of HIV-2 seropositive sera tested while the seronegative control sera were negative in all the dilutions tested. (see Example 19)

Fig. 7 shows the results from fixed dilutions of sera obtained from a panel of HIV-2 seropositive and HIV-2 seronegative donors tested against the peptide coupled to a non-dendritic peptide carrier and alone (see Example 19). The sera were also tested against recombinant protein HIV-2 gp36.

All the seropositive donorsera were reactive in the assay
while no reactivity was detectable with the seronegative
control sera.

Fig. 8 shows dilution curves of sera obtained from one HIV-2
seropositive donor and one HIV-2 seronegative donor tested
against the gp36 peptide alone and coupled to a non-dendritic
peptide carrier. The sera were also tested against recombi-
nant protein HIV-2 gp36. The peptides, when coupled to the
non-dendritic peptide carrier, were recognized at higher
dilutions of the seropositive serum compared to the peptide
alone, see Example 19.

Fig. 9 shows that mice produced antibodies in response to the
derivatized non-dendritic peptide carrier EBA peptide-PPD
conjugate after immunization by antibody production as
described in Example 9. The strongest antibody response was
detectable after 3 immunizations. Absorption to aluminium
hydroxide enhanced antibody production after one immunization
but not after two or three immunizations. (see Example 20)

Fig. 10 shows that mice responded to the EBA peptide after
immunization with the derivatized non-dendritic peptide
carrier EBA peptide alone intraperitoneally or after immuniz-
ation with the derivatized non-dendritic peptide carrier EBA
peptide mixed with Freund's adjuvant subcutaneously. Levels
of antibody reactivities were higher when mixing the branched
peptide construct with Freund's adjuvant. (See Example 20)

Fig. 11 shows that mice responded to the gp120 peptide after
immunization with this peptide derivatized to the non-
dendritic peptide carrier and mixed with Freund's adjuvant
subcutaneously. The strongest antibody response was detect-
able after 3 immunizations. (see Example 20)

Fig. 12 shows percentage inhibition of IL-6 secretion from
human mononuclear cells stimulated with antigens from the
malaria parasite *Plasmodium falciparum* : (————) inhibition

by the beta-2-glycoprotein I peptide coupled to the non-dendritic peptide carrier; (-----) inhibition by the peptide alone. Means of 2 different experiments are shown. (see Example 22)

Fig. 13 shows HPLC analysis of a non-dendritic peptide carrier structure type 2 (see Table 1) derivatized with HIV gp120-peptide. HPLC-analysis of raw product at 280 nm (top) and 220 nm (bottom). "X" is the injection peak, and "Y" is a system peak. "P" is the desired product peak.

Fig. 14 shows that mice produced IgG1 in response to subcutaneous immunization with the EBA peptide derivatized non-dendritic peptide carrier mixed with Freund's complete adjuvant.

Fig. 15 shows that mice produced IgG2a in response to subcutaneous immunization with the EBA peptide derivatized non-dendritic peptide carrier mixed with Freund's complete adjuvant.

Fig. 16 shows that mice produced IgG to the LERLLL HIV-1 gp41 peptide in response to subcutaneous immunization with the LERLLL HIV-1 peptide derivatized non-dendritic peptide carrier mixed with HIV-1 gp120 (aa152-176) peptide derivatized non-dendritic peptide carrier peptide with and without Freund's complete adjuvant.

Fig. 17 shows that mice produced IgG to recombinant HIV-1 gp120 in response to subcutaneous immunization with the HIV-1 gp120 (aa152-176) peptide derivatized non-dendritic peptide carrier mixed with Freund's complete adjuvant.

Fig. 18 shows that mice produced IgG to recombinant HIV-1 gp41 in response to subcutaneous immunization with the LERLLL HIV-1 peptide derivatized non-dendritic peptide carrier mixed with HIV-1 gp120 (aa152-176) peptide derivatized non-

dendritic peptide carrier peptide with Freund's complete
adjuvant.

Fig. 19 shows that mice produced IgG1 to HIV-1 gp120 (aa152-
176) peptide in response to subcutaneous immunization with
the HIV-1 (aa152-176) peptide derivatized non-dendritic
peptide carrier mixed with Freund's complete adjuvant.

Fig. 20 shows that mice produced IgG2a to HIV-1 gp120 (aa152-
176) peptide in response to subcutaneous immunization with
the HIV-1 (aa152-176) peptide derivatized non-dendritic
peptide carrier mixed with Freund's complete adjuvant.

Fig. 21 shows that mice produced IgG1 to the LERLLL HIV-1
gp41 peptide in response to subcutaneous immunization with
the LERLLL HIV-1 peptide derivatized non-dendritic peptide
carrier mixed with HIV-1 gp120 (aa152-176) peptide derivatiz-
ed non-dendritic peptide carrier peptide.

Fig. 22 shows that mice produced IgG2a to the LERLLL HIV-1
gp41 peptide in response to subcutaneous immunization with
the LERLLL HIV-1 peptide derivatized non-dendritic peptide
carrier mixed with HIV-1 gp120 (aa152-176) peptide derivatiz-
ed non-dendritic peptide carrier peptide.

Fig. 23 shows inhibition of TNF secretion in vivo stimulated
by LPS by CKNKEKKC- and KNGMLKGDKVS-derivatized non-dendritic
peptide carriers.

Fig. 24 shows that mice produced IgG in response to three
subcutaneous immunizations with the EBA peptide derivatized
non-dendritic peptide carrier mixed with Freund's complete
adjuvant but that the IgG response was diminished by a fourth
immunization with the EBA peptide derivatized non-dendritic
peptide carrier mixed with Freund's complete adjuvant and
murine recombinant IL-10 compared to a fourth immunization
with the EBA peptide derivatized non-dendritic peptide car-
rier mixed with Freund's complete adjuvant alone.

Fig. 25 shows that mice produced IgG1 to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier mixed with different murine recombinant cytokines or with alumn or with Freund's complete adjuvant.

Fig. 26 shows that mice produced IgG2a to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with murine recombinant TNF or with Freund's complete adjuvant.

Fig. 27 shows that mice produced IgG1 to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with tuftsin or with Freund's complete adjuvant.

Fig. 28 shows that mice produced no IgG2a to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with tuftsin.

Fig. 29 shows that mice produced IgG1 to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with different gamma-interferon specific peptides or with Freund's complete adjuvant.

Fig. 30 shows that mice produced IgG2a to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with different gamma-interferon specific peptides or with Freund's complete adjuvant.

Fig. 31 shows that mice produced IgG1 to the L1 leishmania peptide in response to intraperitoneal immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with different gamma-interferon specific peptides.

Fig. 32 shows that mice produced no IgG2a to the L1 leishmania peptide in response to intraperitoneal immuniz- ation with the L1 leishmania peptide derivatized non- dendritic peptide carrier with different gamma-interferon specific peptides.

Fig. 33 shows that mice produced IgG1 to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with recombinant TNF or a TNF specific peptide or with Freund's complete adjuvant.

Fig. 34 shows that mice produced IgG2a to the L1 leishmania peptide in response to subcutaneous immunization with the L1 leishmania peptide derivatized non-dendritic peptide carrier with recombinant TNF or with Freund's complete adjuvant.

Fig. 35 shows that mice produced IgG1 to the L2 leishmania peptide in response to subcutaneous immunization with the L2 leishmania peptide derivatized non-dendritic peptide carrier with or without a TNF specific peptide or with and without tuftsin.

Fig. 36 shows that mice produced IgG2a to the L2 leishmania peptide in response to one single subcutaneous immunization with the L2 leishmania peptide derivatized non-dendritic peptide carrier with a TNF specific peptide.

Fig. 37 shows that mice produced IgG1 to the HIV-1 gp120 (aa152-176) peptide in response to subcutaneous immunization with the HIV-1 gp120 (aa152-176) peptide derivatized non- dendritic peptide carrier with a TNF specific peptide or with gamma-interferon specific peptides or with tuftsin.

Fig. 38 shows that mice produced IgG2a to the HIV-1 gp120 (aa152-176) peptide in response to one and three subcutaneous immunizations with the HIV-1 gp120 (aa152-176) peptide deriv-

atized non-dendritic peptide carrier with a TNF specific
peptide or with gamma-interferon specific peptides.

Fig. 39 shows that mice produced IgG to the HIV-1 gp120
(aa152-176) peptide in response to subcutaneous immunization
with the HIV-1 gp120 (aa152-176) peptide derivatized non-
dendritic peptide carrier with or without an IL-1 specific
peptide or tuftsin or mixed with Freund's complete adjuvant.

Fig. 40 shows that mice produced IgG to the HIV-1 gp120
(aa152-176) peptide in response to subcutaneous immunization
with the HIV-1 gp120 (aa152-176) peptide derivatized non-
dendritic peptide carrier with or without an IL-1 specific
peptide.

Fig. 41 shows the development of antibodies against Tbp-
peptide after immunizations with peptide conjugated to PPD
and with the peptide coupled to a non-dendritic peptide
carrier (NDPC) with and without use of adjuvant. The analysis
was performed by ELISA.

Fig. 42 shows the development of antibodies against PalA-
peptide after immunizations with peptide conjugated to PPD
and with the peptide coupled to a non-dendritic peptide
carrier (NDPC). The non-coupled, linear peptide was used as
an immunization control. The analysis was performed by ELISA.

Fig. 43 shows the reactivity of different constructs (back-
bone structure numbers refer to Table 1) with a monoclonal
antibody raised against PPD-coupled Tbp-peptide 4. The dif-
ferent constructs were used for coating of the microtiter
plate and then probed with the monoclonal antibody. The
analysis was performed by ELISA.

EXAMPLES

In the examples below, the following abbreviations have been
used:

Standard single-letter and three-letter abbreviations for amino acids.

Aib: alpha amino isobutyric acid.

Alo: allyloxycarbonyl.

BCG: Bacterin Calmette Guerin

Boc: butyloxocarbonyl.

BrAc: bromoacetyl.

CA: citraconic anhydride.

DCC: dicyclohexylcarbodiimide.

DCM: dichloromethane.

Dde: 1-(4,4dimethyl-2,6-dioxocyclohexylidine)ethyl.

DMAP: dimethylaminopyridine.

DMSO: dimethylsulfoxide.

DSS: disuccinimidyl suberate

EBA: erythrocyte binding antigen

EDC: 1-ethyl-3[dimethyl(aminopropyl)]carbodiimide

ELISA: enzyme linked immunosorbent assay

Fmoc: fluorenylmethoxycarbonyl.

HOBt: hydroxybenzotriazole.

HMB: 4-hydroxymethyl benzoic acid.

HMPA: 4-hydroxymethyl phenoxyacetic acid.

IFN: interferon

IL: interleukin

i.p.: intraperitoneally

MSNT: 1-(mesitylene-2-sulfonyl)-3-nitro-1-H-1,2,4-triazole

Mtt: methyltrityl.

NMM: N-methylmorpholine.

NMP: N-methylpyrrolidone.

Palm: palmitoyl residue $(CH_3(CH_2)_{14}CO-)$.

Pbf: 2,2,4,6,7-penta-methyldihydrobenzofuran-sulfonyl

PPD: purified protein derivative from *Mycobacterium tuberculosis*

s.c.: subcutaneously

SPDP: N-succinimidyl 3-(2-pyridyldithio) propionate.

TBTU: 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

TFA: trifluoroacetic acid.

TNF: tumour neurosis factor

Trt: trityl.

Example 1

Amphiphilic parallel α-helix homodimer-forming non-dendritic peptide carrier

<u>Examples of basic chemical strategies</u>

A twenty-amino acid α-N-palmitoylated lipopeptide with a strong α-helix, parallel homodimer-forming tendency was defined based on the heptad-rules detailed above:

palm-AVHKLEHKVAKLEAKGKGKY,

where palm=palmitoyl residue $(CH_3(CH_2)_{14}CO-)$. Histidine residues were included to support helix-formation upon divalent metal-ion chelation, and tyrosine was included as a 280 nm reporter group. The peptide corresponds to two turns of an α-helix with a loosely structured hydrophilic C-terminal stretch (GKGKY).

The synthesis was performed with Novasyn KB resin (Nova-biochem 01-64-0025) containing the base-labile 4-hydroxy-methylbenzoic acid (HMB-) linker for C-terminal attachment of the peptide. All amino acid derivatives were from Novabiochem and all solvents from Merck. The amino acids used were α-amino-Fmoc-protected derivatives with the following side-chain protections: Asn, Cys, Gln, His: trityl (Trt), Lys, Trp: butyloxycarbonyl (Boc), Glu, Asp, Ser, Thr and Tyr: tertbutyl (tBu), Arg: 2,2,4,6,7-pentamethyl dihydrobenzofuran-sulfonyl (Pbf). Peptide synthesis was performed on a Mark-III peptide synthesizer from Schafer-N (Copenhagen) using dedicated software. All operations were performed in a fume hood. The quantitative Kaiser test (Kaiser 1970) was used to detect free amino groups.

Measurement of liberated Fmoc (after piperidine) was done at
301 nm as described by Grant (1992) using the formula
$[E_{301} \times$ dil. $\times$ vol.$]/[10 \times$ weight$_{gram}]$ (μmol/g), where dil. and
vol. are the dilution and the volume, respectively, of the
sample used for measurement. Weight is the weight of the
peptide-resin from which the Fmoc-group is derived.

As an example, 1 g Novasyn KB resin (Novabiochem 01-64-0025)
containing 0.15 mmol/g hydroxyl groups was left in NMP for 20
minutes at room temperature and then washed on a filter with
DCM. The first amino acid (tyrosine) was coupled as an ester
(Blankemeyer-Menge 1990): 300 μmol (=2 times molar surplus)
Fmoc-Tyr(tBu) was mixed with 225 μmol methylimidazole and 300
μmol 1-(mesitylene-2-sulfonyl)-3-nitro-1H-1,2,4-triazol
(MSNT) at 0.2 M in DCM and incubated with the KB-resin in DCM
with shaking for 60 minutes at room temperature (RT). This
coupling was repeated once with new reagents on the same
portion of KB-resin followed by capping with 10 equivalents
of acetic anhydride in the presence of 1 eq. DMAP. Capping
was done for 15 minutes and followed by extensive wash in
NMP. Spectrophotometric determination (301 nm) of the Fmoc-
group cleaved from the coupled tyrosine (cleavage by 20%
piperidine in NMP) gave 134.8 μmol/g resin, which corresponds
to a coupling yield of 89.9%. The Kaiser test performed on a
small sample of resin at this point yielded a deep blue
colour. The rest of the amino acids after this point were
coupled sequentially after activation for 10 minutes (TBTU/-
HOBt/NMM, 1/1/2 molar equivalents compared to amino acid in
NMP) and coupling for 30 minutes in 8 times molar excess,
except isoleucine, threonine, and valine which were double-
coupled for 2 x 30 minutes. The final Fmoc-value corresponded
to 133.9 μmol peptide pr. g resin (original weight). This
corresponds to a yield compared to the first amino acid
coupled of 99.3%. The Kaiser test gave a deep blue colour. At
this point, a small sample of resin was removed for cleavage
of the peptide as a control (peptide before palmitoylation,
Fig. 2A). The rest of the peptide was palmitoylated by a two
times repeated treatment with palmitate as follows: Palmitic

acid corresponding to 10 times the α-amino groups on the peptide-resin was mixed with TBTU, HOBt and NMM (1/1/2 (mol/-mol/mol) corresponding to the palmitic acid) in NMP and activated by shaking at RT for 10 minutes before incubating with the peptide-resin for 30 minutes at RT. After repeated coupling, the Kaiser test yielded a clear colourless solution and a small sample was retained for analysis (peptide after palmitoylation, Fig. 2B). Subsequently, the peptide resin was washed with NMP, 2-propanol, and DCM. To prepare the lipopeptide-resin for attachment of side-chain antigens ("branches"), ε-amino-protecting Boc-groups in lysine were removed by treatment with 6 x 5 ml 50% TFA in DCM for 6 x 30 minutes in a closed bottle with shaking at RT. This was followed by washing the resin on a filter with DCM, 4% NMM in NMP, NMP, water, and ether. At this stage, the peptide-resin was ready for coupling of branch-antigens (see examples below), and the Kaiser test was deep blue. Coupling of a protected amino acid to these attachment points was performed by the normal activation chemistry and yielded a clear peptide-resin by the Kaiser test.

To analyse for the success of synthesis, the two peptide-resin samples (the non-palmitoylated sample and the finished lipopeptide-resin) were treated to remove side-chain protecting groups as above and then cleaved from the resin in the following way:

The peptide resin (100 mg) was treated with 5 ml 1 M NaOH for 10 minutes on ice in an ultrasonic water bath. The resin was then filtered off and the filtrate was collected in 0.1 M acetic acid on ice. This was repeated and followed by 2 times water (MilliQ) incubations for 2 x 10 minutes and 100% acetonitrile in the same way, collecting all filtrates separately in acetic acid. These fractions were then analysed for peptide-yield and purity by HPLC, and relevant fractions were pooled, desalted by preparative reverse-phase HPLC (see Example 5), and freeze-dried. The non-palmitoylated peptide

was predominantly found in the first NaOH-wash, while the
palmitoylated peptide was found in both NaOH-washes.

The products were highly soluble in aqueous solution and were
easily analysed with good purities (>80%) by HPLC. HPLC-
analysis: Solvent A: 0.1% TFA/10% acetonitrile in milliQ
water, solvent B: 0.1% TFA in acetonitrile. Flow: 1 ml/min.
Column: Merck Lichrospher 100, RP 18, 5 μm, 250 × 4 mm. The
gradient was controlled by high-pressure side two-pump mixing
(Jasco PU880 pumps) and ran from 100% A at t=0 min. to 20% A
at t=10 min. and 0% A at t=20 min. to 25 min. followed by a
rise to 100% A at t=26 min. The effluent was monitored at 220
nm on a Varian 9050 detector. Data were collected by Varian
Star chromatography software. Load: 50 μl 1 mg/ml, freeze-
dried, desalted raw-product pool. The main impurities in the
palmitoylated peptide derived from incomplete palmitoylation.
The palmitate-coupled non-dendritic peptide elutes consider-
ably later than the non-palmitoylated control-peptide (in
this HPLC-gradient around 5 minutes later, the HPLC-analysis
is disclosed in Fig. 2). Typical raw product yields were
around 80% and desalting yields also around 80%.

Matrix-assisted Laser-desorption ionization time-of-flight
mass spectrometry of the products yielded a molecular ion
with a mass corresponding well to the expected mass (within 2
D from the expected mass of 2473.1 for the palmitoylated
product, see Fig. 3). Various analyses for the secondary
structure and the aggregation state of the non-dendritic
peptide, including chelation of Zn++- and Co++-ions and
spectrocopy, circular dichroism, tyrosine fluorescence qu-
enching, and gel filtration, are to be performed.

The non-dendritic peptide derivatized resin was washed,
dried, and stored and used directly for standard peptide
synthesis of branch peptides or used for coupling of presyn-
thesized peptides or other whole molecules.

An equally useful peptide was obtained by replacing the double-histidine chelating site using A at these positions.

In another synthesis, I will be used instead of V.

As an alternative to cleavage by NaOH, methanolic ammonia was also employed as this treatment yields the preferred peptide amide.

Also, the peptide will be synthesized using the modified Rink linker (4-(2',4'-dimethoxyphenyl Fmoc-aminomethyl)phenoxy acetic acid) and the amino acid derivatives listed above. In this strategy, Boc will be removed from K by 25% TFA in DCM by a 3 x 10 minutes treatment. This does not affect the binding to the modified Rink linker. This leads to a final, preferred peptide amide product. Also, the cleavage and work-up procedure is simpler than with the 4-hydroxymethyl benzoic acid linker described above. Finally, the modified Rink-linker is stable to hydrazine, allowing extensive use of Dde-protection (see below).

The backbone peptide will also be synthesized with orthogonally protected lysine residues, e.g. Fmoc-K(Mtt)-OH (Mtt=methyltrityl), Fmoc-K(Dde)-OH (Dde=1-(4,4-dimethyl-2,6-dioxocyclohexylidine)-ethyl), Fmoc-K(Aloc)-OH (Aloc=allyloxo-carbonyl), in which side chain protecting groups are removable by 1% TFA, 2% hydrazine and Pd(0)(catalytic hydrogena-tion), respectively. After synthesis, alpha-amino lipid-coupling and selective lysine-side chain demasking, synthesis or coupling of branch peptides or moieties can then be accomplished with no risk of interference from unmasked functional groups in the side chains of other amino acids in the back-bone peptide, especially the carboxylic acid deriving from E.

The change to a selectively cleavable protecting group for side-chain amino groups decreases the heterogeneity in the raw product of the final side-chain derivatized non-dendritic peptide carrier (see Example 5), because of the exclusion of

the possibility of lactam-formation between free side-chain carboxylic groups being activated by TBTU/HOBt/NMM from the branch peptide synthesis and reacting with the deprotected amino groups of the peptide carrier and of the side-chain peptide being synthesized. Also, selective chemistry allows the deprotection of only a part of the lysine epsilon amino groups present, retaining the charge on the lysines taking part in the stabilization of the amphipathic helix (Table 1, structure nos. 2, 3, 5, 6, 7, see below) and/or allowing the inclusion of stimulatory peptides not to be side-chain derivatized (Fig. 1, structure nos. 4 and 5).

As a particular illustrative and preferred example of a combination of linker and protecting group strategy employing selective chemistry, a non-dendritic peptide carrier containing only a single class of attachment sites was synthesized on the acid-labile modified Rink-linker in combination with either hyper acid-labile side-chain amino-protecting groups (methyltrityl- (Mtt-) group, structure nos. 3 and 6) or the hydrazine-labile Dde-group (1-(4,4-dimethyl 2,6-dioxocyclo-hexylidine)-ethyl)(structures 2, 4, 5). The non-dendritic peptide carriers depicted in Table 1, structure nos. 2-7 given below, were synthesized using the following conditions:

All conditions, solvents and reagents as above, except Fmoc-Lys(Dde)-OH and Fmoc-Lys(Mtt)-OH, which were from Novabiochem (nos. 04-12-1121 and 04-12-1137, respectively). For the coupling of the first (C-terminal) amino acid, the modified Rink-resin (4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl) phe-noxyacetamido-norleucyl methylbenzhydrylamine polystyrene), Novabiochem 01-64-0037) was treated with 20% piperidine in NMP for 2 x 5 minutes, washed with NMP and coupled with the amino acid in a molar surplus of 8 times using TBTU/HOBt/NMM activation for 10 minutes and coupling for 30 minutes at RT. After a second coupling using the same conditions, the coupling yields were typically from 80-90%. After capping with acetic anhydride/DMAP in NMP (20 equivalents of acetic anhydride, 1 equivalent of tertiary amine (DMAP or NMM), 10

minutes) followed by thorough washing by NMP automated syn-
thesis was performed as above. After cleavage of the final
Fmoc-group, palmitic acid was introduced as above and,
subsequently, lysine side-chain protecting groups were
removed:

Mtt: 1% TFA in 5% triisopropylsilane in DCM, 3 x 5 minutes
treatment. Liberated Mtt was monitored at 470 nm.

Dde: 2% hydrazine in NMP, 3 x 5 minutes, liberated Dde was
monitored at 290 nm.

The yield of side-chain attachment points were generally from
60-80 %.

After this, to analyse for the purity of the non-dendritic
peptide carriers, a small portion of the peptide-resins was
cleaved by the standard TFA-cleavage procedure (95 % TFA in
water, no scavengers added, 3 x 30 minutes treatment in
closed container, followed by precipitation in cold diethyl-
ether, filtration, dissolution in water and freeze-drying)
and analysed by analytical reverse-phase HPLC.
Peptides were of good purities and yields, and mass spectro-
metry of the main raw product peak is expected to give mol-
ecular weight values not differing more than 5 Da from the
theoretical molecular weights given in Table 1.

Synthesized structures

The two types of model structures depicted in Table 2 have
been synthesized with Mtt-protection to verify structure by
MS-analysis. Mtt was removed by 1% TFA/5% triisopropylsilane
in DCM, and a branch-peptide (YLEN and WNSG, respectively)
was synthesized on half of each backbone. After cleavage and
purification, all constructs will be analysed by MALDI-TOF
mass spectrometry. The mass spectrometry analysis is expected
to give molecular weights not differing more than 5 Da from
the theoretical values depicted in Table 2.

A number of heptad-containing model non-dendritic peptide
carriers will also be synthesized, in which two types of
protections for lysine were employed; a selectively cleavable
one for attachment-point lysines, which includes only lysines
at the "c"-positions in the heptads and Boc-protected lysines
at the "g"-positions. This allows derivatization at the "c"-
positions and, at the same time, retention of charge at the
"g"-positions. Also, another design will be used in which an
additional selectively deblockable K-residue will be intro-
duced at the f-positions. Thus, the following model peptides
will synthesized with the selectively deblockable lysine
residues being denoted K:

VAKLEAKVAKLEAK (1)

VAKLEAKVAKLEAK (2)

VAKLEKKVAKLEKK (3)

These syntheses will be performed on a modified Rink-linker
as described above. After deblocking the K-residues, they
will be derivatized with AELGGQFHHKSENG, cleaved, and worked
up and analysed for alpha-helix content as well as for homo-
dimer formation compared to the unsubstituted model peptide.
Accessibility and antigenicity of the attached peptide will
be analysed with a monoclonal antibody raised against the
peptide.

Helix- as well as homodimer-formation is expected to decrease
in the derivatized peptide (2) compared to the two other
peptides, which are expected to be good alpha-helix- and
dimer-formers even after side-chain derivatisation. Also,
peptides (1) and (3) are expected to present the side-chain
peptides more efficiently to the monoclonal antibody, result-
ing in a more efficient recognition in an indirect ELISA than
peptide 2.

Other variants

By combining three different side-chain protection labilities
(weak acid, stronger acid, and hydrazine), it was possible to

synthesize non-dendritic peptide carriers employing both
"normal" side-chain attachment points as well as special
attachment points for coupling stimulatory peptides, e.g.
cytokine peptides (see Example 5) (see Table 1, structure 7).

A range of other standard peptide carrier variants will also
be synthesized and analysed in a similar way; these variants
include a peptide carrier that is lactam-stabilized by sub-
stituting the histidine pair with a lysine-glutamic acid pair
and, subsequently, allowing their side-chains to react by
selectively deprotecting the side-chains; another variant is
substituted with secondary structure nucleators at the attac-
hment points; yet another variant will be synthesized on a
dibranched linker molecule, yielding a non-dendritic peptide
consisting of two peptide chains held together at their C-
terminal; also, a variant with the tripalmitate structure of
Fig. 3 instead of a linear palmitate will be synthesized.

Finally, a non-dendritic carrier peptide in which the
palmitate is attached by a thioester linkage to the side-
chain thiol group in a N-terminal cysteine will be syn-
thesized and used for immunization experiments.

Table 1	
Types of non-dendritic peptide carriers. Molecular weights (mw) are given for the unprotected peptides.	
1	Palm-AVH*K*LEH*K*VA*K*LEA*K*G*K*G*K*Y-OH (*K*=K(Boc)), <u>mw.:2473.1</u>
2	Palm-AVH*K*LEH*K*VA*K*LEAKG*K*G*K*Y-NH$_2$ (*K*=K(Dde)), <u>mw.:2472.1</u>
3	Palm-AVH*K*LEH*K*VA*K*LEAKG*K*G*K*Y-NH$_2$ (*K*=K(Mtt)), <u>mw.:2472.1</u>
4	Palm-AVH*K*LEH*K*VA*K*LEAKG*K*G*K*YVQGEESNDK-NH$_2$ (*K*=K(Dde)), <u>mw.:3459.1</u>
5	Palm-AVH*K*LEH*K*VA*K*LEAKG*K*G*K*YVQGEESNDK-NH$_2$ (*K*=K(Dde)), <u>mw.: 3459.1</u>
6	Palm-AVA*K*LEAKVA*K*LEAKG*K*G*K*Y-NH$_2$ (*K*=K(Mtt)), <u>mw.:2340.0</u>
7	Palm-<u>*K*</u>VA*K*LEAKVA*K*LEAKG*K*G*K*Y-NH$_2$ (<u>*K*</u>=K(Dde) and *K*=K(Mtt)), <u>mw.:2397.1</u>

Table 2	
Types of model non-dendritic peptide carriers	
1	*K*G*K*G*K*GL-NH2 palm-*K*G*K*G*K*GL-NH2
2	*K*GKG*K*GL-NH2 palm-*K*GKG*K*GL-NH2
3	KG*K*G*K*GL-NH2 palm-KG*K*G*K*GL-NH2
	K=Lys(Boc) *K*=Lys(Mtt). palm=palmitoyl residue Molecular weights (mw) are given for the unprotected peptide. <u>Mw.:</u> 685.9 (non-palmitoylated peptides) 924.3 (palmitoylated peptides) Branch-peptides: YLEN- Resulting molecular weights: 1732.9 (2 attachment points, non-palmitoylated) 1971.4 (2 attachment points, palmitoylated) 2256.6 (3 attachment points, non-palmitoylated) 2495.0 (3 attachment points, palmitoylated)
4	GKGKGKLYL, where K=Lys(Mtt) mw (unprotected): 962.2
5	Palm-GKGKGKLYL, where K=Lys(Mtt) mw (unprotected): 1200.6
6	N^ϵ[WNSG]$_3$-palm-GKGKGKLYL, where K=Lys(Mtt) mw (unprotected): 2534.0

Example 2

**A solid-phase for the simultaneous synthesis but separate
release of derivatized non-dendritic peptide carriers as well
as free peptides**

The following method was devised for the purpose of obtain-
ing, directly by solid-phase synthesis, both a non-dendritic
peptide carrier (NDPC) coupled peptide and the same peptide
in a free form that allows direct inspection of the success
of the synthesis and gives access to a control-peptide for
immunization experiments, for characterization of antibodies
etc.

This can be achieved by substituting a solid-phase resin with
a mixture of orthogonally labile linkers, e.g. an acid-labile
linker as 4-hydroxymethylphenoxy acetic acid (HMPA) combined
with a base-labile 4-hydroxymethylbenzoic acid (HMB). Then
one of the linkers is blocked temporarily, while a non-
dendritic peptide carrier (NDPC) is synthesized on the other
linker. The treatment that removes NDPC side-chain protecting
groups also removes the protecting group on the other linker.
In an example of such a synthesis, a Novasyn base resin will
be partially substituted by the HMPA-linker by incubation of
the resin with TBTU/HOBt/NMM-activated linker in a 0.1 molar
ratio to the number of amino groups on the resin, coupling in
NMP for 1 x 30 minutes at room temperature under shaking.
Then the resin will be washed with NMP, and the remaining
amino groups will be blocked by a 5 times molar surplus of
Fmoc-norleucine activated as above for 2 x 30 minutes at room
temperature with shaking. Following a wash in NMP, Boc-
glycine wi`` be coupled to the free hydroxyl-group of the
HMPA-linke. by a mesitylene/imidazole coupling (see Example
1). After wash with NMP, the α-amino group of the Fmoc-L will
be removed by 20% piperidine in NMP. After yet another NMP-
wash, the HMB-linker will be attached by activation and
coupling twice as described above with 5 times molar surplus.
The NDPC will be synthesized on this solid phase as detailed

in Example 1. Side-chain protecting groups as well as the
Boc-G coupled to the HMPA-linker will be cleaved by 95% TFA
in water. HMPA is now ready for coupling another amino acid
as an ester. The resulting solid phase can be used for the
simultaneous synthesis of the free peptide and the peptide-
derivatized NDPC, the former being liberated and deprotected
in one step by TFA-treatment (95% TFA in water), while the
derivatized NDPC is only released by base-treatment (see
Example 1).

In another synthesis to be performed, the resin will be
partially substituted by a Fmoc-protected RINK-linker by
incubating the resin with TBTU/HOBt/NMM-activated linker in a
0.1 molar ratio to the number of amino groups on the resin,
coupling in NMP for 30 minutes (once) at room temperature
with shaking. Subsequently, the HMB-linker will be coupled by
activation as above to the rest of the available amino groups
on the resin coupling twice (30 minutes, room temperature,
shaking) with 5 times molar surplus. Now the Fmoc-group is
removed from the Rink-linker by 20% piperidine/NMP, and a
Boc-G is coupled to the free amino group by TBTU/HOBt/NMM
activation chemistry. These coupling conditions favour coup-
ling to the amino groups compared to esterification to the
hydroxyl-groups of the HMB-linker. The NDPC is now syn-
thesized on this solid phase as shown in Example 1. In the
deprotection step with 50% TFA in DCM, the Boc-groups pro-
tecting the ϵ-amino group of the backbone lysines as well as
the Boc-group of the Rink-linker-bound G will be removed. The
resulting solid phase can be used for the simultaneous syn-
thesis of the free peptide and the peptide-derivatized NDPC,
the former being liberated and deprotected by extensive TFA-
treatment (95% TFA in water), while the derivatized NDPC is
only released by base-treatment (see Example 1). The free
peptide will be extended in the carboxylic end by one glycine
residue.

Example 3

Non-dendritic peptide building blocks for creating looped peptides

Loops are very common and easily distinguishable features of most proteins of which the tertiary structure is known. Moreover, such loops often constitute immunodominant parts of the protein, and a method for their easy and general prepara-tion in a loop-inducing set-up is therefore highly interest-ing. As an example, this can be done by synthesizing a first non-dendritic building block, palmitoylated tetrapeptide PC(Trt)K(Palm)L, on a Novasyn KB resin by the solid-phase method detailed in Example 1. The Fmoc-group will be left on K, and the ϵ-amino group will be deprotected by treatment with 50% TFA in DCM as in Example 1. Subsequently, palmitic acid will be coupled to this amino group as described in Example 1, the completeness of coupling being followed by the Kaiser test. Hereafter, the α-amino Fmoc-group will be cleaved and the rest of the peptide will be synthesized. The peptide will be left on the resin after cleavage of the last Fmoc-group. The other building block could be an identical peptide but soluble and cleaved from the resin by NaOH-treat-ment as described in Example 1, but not deprotected by acid and still containing the last Fmoc-group. As another example of the soluble building block, Palm-PC(Trt)LG will also be synthesized. As yet another example of this soluble building block, Palm-PK(BrAc)LB will be synthesized, in which BrAc is a bromoacetyl moiety introduced by reacting the ϵ-amino group of the K residue with bromoacetic acid after side-chain deprotection with TFA/DCM. The target-peptide to be presented as a loop (target peptides to be included will be peptides corresponding to loops in human IL-1β (LQGQDMEQQV (aa31-40 in the circulating form of the protein), DPKNYPKKKMEKRF (aa86-99 in the circulating form of the protein), VQGEESNDK (aa47-55 in the circulating form of the protein), GGTKGGQDIT (aa135-144 in the circulating form of the protein and the correspon-

ding loops in porcine interleukin-1β) are now synthesized on
the free alpha-amino group of the solid-phase bound peptide
by standard methods (Example 1). In target peptides contain-
ing cysteine, target peptide C will be protected orthogonally
to C(Trt). After cleavage of the last Fmoc-group of this
solid-phase bound peptide, the free building block will be
activated in a big molar surplus (10 times) and coupled with
TBTU/HOBt/NMM in NMP to the solid-phase bound peptide as
described in Example 1 for palmitic acid until the Kaiser
test is negative. The Fmoc-group and side-chain protecting
groups will be removed as described in Example 1, and cycliz-
ation on the resin will be done by oxidation with I_2/NMP
until a negative Ellman-reaction (Ellman, 1959). With the
bromoacetylated building block, cyclization occurs in PBS, pH
7.2, on the resin with a simultaneous release of HBr, forming
a stable thioether. Remaining Cys-protecting groups, if any,
will be removed by their specific treatment, after which the
whole complex will be liberated by NaOH as above. Disulfide-
formation may also be performed in solution after liberation
of the linear peptide by using low concentration of the
peptide under disulfide-forming conditions (see e.g. Grant
1992). Other functional group pairs may be used in analogy
with the methods described above, as long as any potentially
interacting groups in the loop peptide are orthogonally
protected. Examples to be perfomed include lactam-bridge
formation between glutamic acid or aspartic acid and lysine.
After synthesis and cleavage, peptides will be analysed on
HPLC as described in Example 1. Cyclic peptides are expected
to elute before the corresponding linear ones.

Example 4

Anchoring peptide.

The HMB-linker allows the preparation of peptide hydrazides
(C-acyl-hydrazines) by cleavage with hydrazine. Peptide
hydrazide is an excellent reagent for coupling mildly oxi-
dized or reducing carbohydrates or carbohydrate-containing

compounds, e.g. glycoproteins. An example of the possible
synthesis of such a peptide is as follows:

A peptide, such as the ones listed below, will be synthesized
as described in Example 1 and derivatized with α-N-terminal
palmitic acid before deprotection with 50% hydrazine-hydrate
in dioxane/methanol (9/1) for 2 hours at 4°C, followed by
filtration, wash in the same solvent and neutralization of
the filtrate, evaporation, and finally dissolution in water
and lyophilization (NovaBiochem 1994). The peptide will be
selected from the following:

- a general T-cell stimulatory peptide such as QYIKANSKFIG-
  ITE (tetanus toxoid 830-843), FNNFTVSFWHRVKVSASHLE (teta-
  nus toxoid 947-967), DQVHFQPLPPAVVKLSDALI (*Mycobacterium
  tuberculosis* 38 kD antigen 350-369), DIEKKIAKMEKASSV-
  FNVVNS (*Plasmodium falciparum* circumsporozoite protein
  378-398), KLLSLIKGVIVHRLEGVE (measles virus F-protein
  286-302), LDNIKGNVGKMEDYIKKNNK (*Plasmodium falciparum*
  MSP-1, 260-279), and polyepitope constructs consisting of
  a linear covalent arrangement of a number of different T-
  cell epitopes without flanking sequences (see Thomson
  1995);
- a generally stimulating peptide such as tuftsin oligomers
  [TKPR]$_N$, in which N is preferably from 2-5, or muramyl
  dipeptide (N-acetyl-muramyl-L-alanyl-D-isoglutamine);
- a spacer peptide, providing detectability by its content
  of Y, as in palm-YGLAELKG;
- a combination of a peptide derivatized non-dendritic
  carrier prepared as described in Examples 5 and 6, but
  liberated by hydrazine to yield the peptide hydrazide, in
  which the included branch peptides are constituted by any
  of the T-cell stimulatory peptides mentioned above.

For peptides not containing Y or W, one Y will preferentially
be inserted at one of the ends of the peptide. Reversed phase
HPLC-analysis of a standard backbone peptide as described in
Example 1, compared with an analysis of the same peptide

cleaved as a hydrazide, will presumably show very little difference between the behaviour of these two peptides in HPLC, both with regard to retention time and purity. Mass spectrometry is expected to show the generation of peptides increased by 14 D, corresponding to the substitution of -COOH by -CONHNH$_2$. The hydrazide anchoring peptide will then be desalted, freeze-dried, and used for conjugating a carbohydrate with accessible carbonyl groups either obtained by controlled mild oxidation by periodate or provided by the reducing end of the carbohydrate (see Example 10).

Example 5

Attachment of peptides by direct, sequential chemical solid-phase synthesis on a non-dendritic backbone peptide carrier

The non-dendritic backbone peptide carrier coupled to the solid phase can be used directly for conventional solid-phase peptide synthesis. One way of doing this is to use conventional sequential petide synthesis methodology, using Fmoc-based chemistry on base- or acid-cleavable backbone-peptides of the types described in Example 1. After synthesis of branch-peptides, protecting groups are removed by TFA, in the case of the modified Rink-linker, simultaneously cleaving the derivatized non-dendritic peptide carrier from the solid-phase, and, in the case of the base-cleavable HMB-linker, followed by standard cleavage of the branched peptide complex by aqueous base and work-up by desalting and freeze-drying. As an example of this, a synthesis was performed in the following way:

A Novasyn KB resin derivatized with the backbone lipopeptide of Example 1 (Table 1, structure 1) was estimated to contain a maximum of 6 times the original amino groups as 6 ϵ-amino groups are introduced at each amino group on the resin. 150 mg derivatized resin, corresponding to 100 mg original resin, was used for synthesis using standard Fmoc-chemistry and TBTU/HOBt/NMM preactivation as described in Example 1. After

synthesis, the last Fmoc-group and side-chain protecting
groups were removed, and the whole branched peptide complex
was cleaved from the solid phase by the standard treatments
described in Example 1. Peptide-containing fractions were
desalted as a pool by semi-preparative chromatography on a
Waters PrepPak 25 x 10 cartridge holder containing a radially
compressed Delta-Pak C18 300 Å cartridge at 10 ml/min using
two Jasco PU880 pumps and a step-gradient, eluting at 100%
acetonitril/TFA, collecting the complete peptide peak and
freeze-drying. The freeze-dried material was highly soluble
in aqueous buffers. HPLC analysis showed that the branched
lipopeptide complex had a lower retention time than the free
lipopeptide-backbone alone. Mass spectrometry as well as a
calculation of the actual number of branches obtained in the
synthesis were performed. The calculation being based on the
E_{280} of a known concentration of the branched peptide, com-
pared to its theoretical Y/W-content and molecular weight,
will be performed.

As a particularly preferred example, a modified Rink-MBHA-
resin was used for the synthesis in combination with the
selective chemistries mentioned in Example 1, i.e. Mtt-
and/or Dde-protected lysine in addition to standard Boc-
protected lysine. The principles of branch-peptide synthesis
are as above, the only difference being the simpler TFA-
cleavage and work-up procedure (see Example 1) and, of
course, that attachment points are created by selective
deblocking.

On backbone no. 1 (Table 1), when nothing else is indicated:

Malarial peptides:
    TLTKEYEDIVLKSHMNRESDD (EBA peptide)
    LKSHMNRESDDGELYDENS (EBA peptide)

Peptides from Ag332 from *Plasmodium falciparum*:
    (VTEEI)
    ([VTEEI]$_2$)
    ([VTEEI]$_3$)

Leishmania peptides: (on backbone no. 6 (Table 1))
    YDQLVTRVVTHEMAHA
    EAEEAARLQA

HIV peptides
    LERLLL (HIV gp41 peptide)
    GEIKNCSFNISTSIRGKVQKEYAFF (HIV gp120 peptide)
    GEIKNCSFNISTSIRGKVQKEYAFF on backbone 2, HPLC-analysis in
    Fig. 13.
    GEIKNCSFNISTSIRGKVQKEYAFF on backbone 4
    GEIKNCSFNISTSIRGKVQKEYAFF on backbone 5
    GEIKNCSFNISTSIRGKVQKEYAFF on backbone 6
    WGCSKLICTTAVPWN (HIV gp41 peptide)
    LQDQARLNSWGCAFRQVCHT (HIV gp36 peptide)

Actinobacillus pleuropneumoniae peptides
    Tbp-2 (Transferin-binding protein type 2)
    SGGKGSFDLEDV (peptide 1)
    AELGGQFHHKSENG (peptide 4)

    PalA (proteoglycan-associated lipoprotein)
    GMTAEDLQTRYN (peptide 1)
    TEADYAKNRAVLEY (peptide 5)

Peptides from Salmonella induced filament hypothetical pro-
tein, selected by hydrophilicity:
    CASQRDRFQVHNPHENDA
    CKSQSGIEKTTRILHHANISESTQQN
    CQATAKMAEEQLTTLHVRSEQQS

More complex structures were synthesized in which the non-
dendritic peptide carrier contained two or more different

side-chain protecting groups for lysine ε-amino groups. As an
example of the use of a non-dendritic peptide carrier carry-
ing three different types of side-chain protecting groups,
structure no. 7 in Table 1 was synthesized. After palmitoyla-
tion, side-chain Mtt-groups were removed by 1% TFA/5% TIS in
DCM, followed by attachment of branch peptides either by
sequential synthesis or by en bloc couplings. The alpha-amino
groups of the side-chain peptides were acetylated with acetic
anhydride. Hereafter, the Dde-group was removed by 2%
hydrazine in NMP and the liberated amino-group was derivatiz-
ed with SPDP. Finally, this was coupled with a cysteine-
extended peptide.

The following more complex structures were synthesized.
Numbers refer to the non-dendritic peptide carrier structures
in Table 1. Lys(Dde) and Lys (Mtt) denote lysine residues
protected by Dde and Mtt respectively during synthesis.

Mw.= Molecular weight

{[N^{ϵ}(IL-1ßpep-C-Cys)Lys(Dde)][N^{ϵ}(gp120)Lys(Mtt)]$_4$}-
Structure 7, mw.: 14862,

{[N^{ϵ}(IL-1ßpep-N-Cys)Lys(Dde)][N^{ϵ}(gp120)Lys(Mtt)]$_4$}-
Structure 7, mw.: 14862,

{[N^{ϵ}(IFNγ-1)Lys(Dde)][N^{ϵ}(gp120)Lys(Mtt)]$_4$}-
N^{α}[Lys(Dde)]-Structure 6, mw.: 18573,

{[N^{ϵ}(IFNγ-2)Lys(Dde)][N^{ϵ}(gp120)Lys(Mtt)]$_4$}-
N^{α}[Lys(Dde)]-Structure 6, mw.: 18604,

{[N^{ϵ}(TNFα)Lys(Dde)][N^{ϵ}(gp120)Lys(Mtt)]$_4$}-
N^{α}[Lys(Dde)]-Structure 6, mw.: 15231,

{[N^{ϵ}(IFNγ-1)Lys(Dde)][N^{ϵ}(Leishmania 1)Lys(Mtt)]$_4$}-
N^{α}[Lys(Dde)]-Structure 6, mw.: 14594,

{[N^ϵ(IFNγ-2)Lys(Dde)][N^ϵ(Leishmania 1)Lys(Mtt)]$_4$}-
N^α[Lys(Dde)]-Structure 6, mw.: 14625,

{[N^ϵ(TNFα)Lys(Dde)][N^ϵ(Leishmania 1)Lys(Mtt)]$_4$}-
N^α[Lys(Dde)]-Structure 6, mw.: 11252,

{[N^ϵ(IFNγ-1)Lys(Dde)][N^ϵ(Leishmania 2)Lys(Mtt)]$_4$}-
N^α[Lys(Dde)]-Structure 6, mw.: 11462,

{[N^ϵ(IFNγ-2)Lys(Dde)][N^ϵ(Leishmania 2)Lys(Mtt)]$_4$}-
N^α[Lys(Dde)]-Structure 6, mw.: 11493,

{[N^ϵ(TNFα)Lys(Dde)][N^ϵ(Leishmania 2)Lys(Mtt)]$_4$}-
N^α[Lys(Dde)]-Structure 6, mw.: 8120,

where

IL-1ßpep: VQGEESNDK (I1-1$_\beta$(163-171))
IL-1ßpep-C-Cys:VQGEESNDKC
IL-1ßpep-N-Cys:CVQGEESNDK
IFNγ1(1-39): HGTVIESLESLNNYFNSSGIDVEEKSLFLDIWRNWQKDG(C)
IFNγ2(95-133): AKFEVNNPQVARAAFNELIRVVHQLLPESSLRKRKRSRC
TNF-α(70-80): (C)PSTHVLLTHTI
gp120: GEIKNCSFNISTSIRGKVQKEYEAFF
Leishmania 1: YDQLVTRVVTHEMAHA
Leishmania 2: EAEEAARLQA

(C) denotes a cysteine residue, not part of the natural
sequence, added for the purpose of coupling through SPDP (see
Example 7).

The peptides will be analysed for purity by HPLC and the
molecular weight will be analysed by mass spectrometry. The
raw products are expected to contain easily identifiable and
purifiable main peptide products (>60%) conforming to the
molecular weights given above within 5 Da.

As examples of planned syntheses, the following peptides will
be synthesized on a number of backbones including the types
no. 3, 5, 6, and 7 depicted in Fig. 1:

[QGPGAP]$_4$ (malarial circumsporozoite protein)

GHPLQKTY (band-3 peptide)

LTPLEELYP (band-3 peptide)

KNGMLKGDKVS (β_2-glycoprotein-I peptide)

CKNKEKKC (β_2-glycoprotein-I peptide)

Peptides from human and porcine IL-1ß, selected by hydro-
philicity and homology considerations:

    See Example 3.

Other backbones will contain auxiliary peptides selected from
cytokine peptides and from the promiscous T-cell binding
peptides included in the list in example 4. In particular,
tuftsin-peptides ([TKPR]$_N$, with N=3-7) and the IL-1ß nonapep-
tide VQGEESNDK will be included in a number of linear and
branched arrangements (in addition to the linear inclusion of
the IL-1ß-peptide in peptide structure no. 5 (Table 1)).

In addition, further examples of synthesized peptides are
mentioned in the examples below.

All constructs will be analysed by mass spectrometry and
reverse phase HPLC for molecular weight and purity. This will
also be done for all constructs mentioned in the following
examples.

Low loading of backbone peptide

To investigate the influence of the density of the non-
dendritic backbone peptide carrier in the solid phase on the
performance as a scaffold for coupling branch peptides, two
deliberately low-substituted solid-phase complexes were
prepared, one at 31 μmol/g (15 minutes coupling of first
amino acid) the other at 61 μmol/g (30 minutes coupling of
first amino acid), and both compared to the standard substi-

tution of around 130 μmol/g as in Example 1 on a Novasyn KB
resin (Novabiochem). In both cases, additional attachment
points on the solid phase resin were blocked by acetic
anhydride/DMAP before further synthesis. A model peptide
GHPLQKTY was coupled to the non-dendritic backbone peptide
solid phase complex by direct synthesis as above, and the
Kaiser test was performed and the Fmoc value measured at each
step throughout synthesis. It was observed that the maximum
number of branches were more easily coupled with higher
homogeneity with the low-loaded solid-phase complex.

Direct coupling to multiplied attachment points

Multiplied attachment points are introduced by coupling
α-Fmoc-K(ε-Fmoc) to the non-dendritic backbone peptide car-
rier amino groups. A multiplied attachment point may be
introduced at any point during synthesis of branch-peptides,
including after the first lysine, to provide a dendritic
aspect to the attachment point. Coupling concentration of
amino acids and coupling reagents should be adjusted accord-
ing to the increased number of equivalents. Prolonged coup-
ling times may be necessary in order to overcome the
increased "crowdedness" of the backbone.

In the present example, a two turn α-helical amphipathic
peptide containing Y [VYKLEAKVAKLEAK] will be synthesized on
a normal non-dendritic peptide carrier backbone as well as to
a double-attachment point substituted backbone by the methods
outlined above. Fmoc-values will be determined and the Kaiser
test will be performed after all coupling steps. The E_{280} of
the final product will also be measured and compared to the
theoretical value derived from the knowledge of the number of
tyrosine residues in the molecule (see Perkins 1986). Final-
ly, mass spectrometry and circular dichroism will be per-
formed. The results are expected to show that the double-
density coupling was possible by selecting the appropiate
reaction conditions and that couplings progressively get
harder to drive to completion as the length of the peptide

increases. Circular dichroism of the final, cleaved and
dissolved product is expected to reveal an increased relative
amount of α-helix compared to the normal derivatized non-
dendritic peptide carrier.

Coupling of two different peptides to the same backbone.

1. By use of multiplied attachment points:

This can be accomplished by using lysine with an ε-amino
protection group that is stable both to TFA and to
piperidine. One such group is Dde ((1-(4,4-dimethyl-2,6-
dioxocyclohexylidine)-ethyl) which is cleaved by 2%
hydrazine in NMP. Another such group is Aloc (allyloxy-
carbonyl) which is cleaved by Pd(0)-catalyzed hydrogena-
tion. These lysine derivatives are both commercially
available. Coupling this amino acid to a non-dendritic
backbone peptide and deprotecting the Fmoc-group with
piperidine provides α-amino-groups for coupling of the
first type of branch, which should be terminated with a
Boc-α-aminoprotected amino acid. Then by 5 × 3 minutes
treatment with 2% hydrazine hydrate in NMP, followed by
NMP-wash, the ε-amino-functionality is available for
coupling the other branch. The reverse order of oper-
ations is not possible, as Fmoc is not stable to
hydrazine. Also, the HMB-linker is hydrazine-labile, but
a short-time, low level exposure in an aprotic solvent
like NMP probably does not affect the HMB-linker.

2. Inclusion of additional peptides in the backbone part of
the non-dendritic peptide carrier or as side chains or parts
of side chains:

Using the principles outlined above, different peptides
or polypeptides may be included in a branched peptide
complex using a non-dendritic backbone peptide, intro-
duced by

-differently protected attachment points (two different peptides).

-linear combinations of different antigens in the branches.

-linear combinations of different antigens at the N- and/or C-terminals of the backbone-peptide.

If included in the backbone peptide, such additional peptides are adequately protected to avoid side-chain substitution in those parts of the backbone. For positioning at an orthogonal attachment point, the peptide should also be left in a protected state. If such a peptide is of universal use, it may be included in a general, pre-synthesized "branched backbone" structure. In one embodiment, the non-dendritic backbone peptide according to Example 1 was synthesized with a C-terminal extension consisting of the IL-1-β peptide VQGEESNDK, with a Dde-protected lysine (see Example 1, Table 1, structure nos. 4 and 5). This backbone peptide was then used to couple the antigenic HIV gp120 peptide, GEIKNCSF-NISTSIRGKVQKEYAFF (see Example 5) by direct synthesis and compared with the same construct without the IL-1-β peptide extension in the immunization experiment in examples below.

In the present example, using the non-dendritic backbone peptide disclosed in Example 1, a T-cell stimulatory peptide included in the list given in Example 4 will first be synthesised on the α-amino group, followed by palmitoylation and synthesis of an antigenic peptide on the ε-amino groups.

One such synthesis will be performed with the T-cell stimulatory peptide QYIKANSKFIGITE (Tetanus toxoid 830-843) on the α-amino group and GEIKNCSFNISTSIRGKVQKEYAFF (HIV gp120 peptide) as the antigenic peptide coupled to the ε-amino groups. In another synthesis, the same

antigenic peptide will be coupled in combination with the
IL-1-β-derived stimulatory peptide VQGEESNDK.

Example 6

**Indirect coupling: Attachment by coupling chemically presyn-
thesized, blocked peptides.**

Please note that while these examples show only syntheses
employing single-attachment points, similar couplings are
possible on multiple attachment points and differently pro-
tected attachment points as long as the introduction of
additional, unblocked functional groups is avoided.

Coupling in the "N-terminal out" orientation

In an example synthesis, the peptide to be coupled will be
synthesized, using Fmoc-chemistry on Novasyn KB, and cleaved
from this solid-phase in its side-chain, and α-amino blocked
form by aqueous base treatment leaving out piperidine- and
TFA-treatments. This blocked peptide will be used directly
without further purification, preactivated by TBTU/HOBT/NMM
in NMP, and subsequently incubated in 10 times molar excess
with the backbone peptide on the solid phase, until the
Kaiser test is negative. To obtain complete coupling, rather
than prolonging the coupling time for more than one hour,
coupling will be repeated with a new portion of activated,
blocked peptide.

Coupling in reverse orientation

In this example, the peptide to be coupled will be syn-
thesized on the very acid-labile 3-(amino-4-methoxybenzyl)-
4,6-dimethoxyphenyl-propionic acid linker coupled to a Nova-
syn base solid-phase, including a final piperidin-cleavage of
the α-amino Fmoc-group, yielding the side-chain protected
peptide as the amide.

This peptide will be coupled to the amino groups of a solid-phase bound standard non-dendritic backbone peptide carrier by glutaraldehyde or by EDC (see Example 7), washed, cleaved, and purified.

Example 7

Indirect coupling: Attachment by coupling chemically presynthesized or recombinantly expressed, side-chain non-blocked peptides

Here some examples on chemoselective ligation chemistries for unprotected peptides, being synthetic, or natural (proteolytic fragments, recombinant fragments, etc.), are presented. In the special case of synthetic or recombinant peptides, the freedom of being able to include specific amino acids, especially cysteine, at any desired position in the peptide chain greatly facilitates such couplings and expands the range of coupling strategies available. However, methods do exist to chemoselectively couple unprotected peptides of any sequence.

Coupling to standard backbone of unmodified peptide by temporary protection

Preferred temporary protection methods include citraconylation (primary amino group protection, the protecting group being released by low pH) and Fmoc-derivatization by Fmoc-succinimide (primary amino group protection, the protecting group being released by piperidine).

In one example, presynthesized peptides will be dissolved to 2 mg/ml in 0.1 M NaHCO$_3$, pH 9, and 10 μl pr ml citraconic anhydride (CA) will be added, and the pH readjusted to 9 with 1M NaOH, followed by another addition of CA. This will be repeated until the pH remained stable upon addition of CA. Incubation will then be performed overnight at 4^{0}C. Then 10 mg EDC (1-ethyl-3 [dimethyl (aminopropyl)] carbodiimide,

(from Pierce Ltd.)) will be added per mg peptide and incu-
bated for 10 minutes before addition of a non-dendritic
backbone peptide carrier, still attached to the solid phase.
Subsequently, this suspension will be allowed to react under
agitation for 2 hours at room temperature. The product will
be worked up, using the procedure outlined in Example 1
(citraconic anhydride groups are removed by acid).

In another example, temporary protection will be achieved by
Fmoc, introduced as the succinimide (1 equivalent) to a 1 M
solution of the peptide in carbonate (1 equivalent) in
water/acetone (1/1, vol/vol) incubating overnight under
stirring. Then pH will be adjusted to 2 with conc. HCl and
acetone will be removed *in vacuo*. The product will be taken
up in chloroform and washed with 0.1 M HCl and recovered from
the organic phase.

Coupling to standard backbone of Cys-substituted peptide by chemoselective ligation

Cysteine can be included at any position in a synthetic or
recombinant peptide as long as it does not interfere with the
reactivity of the peptide. It can, of course, also be intro-
duced in the backbone peptide, preferably by attaching it to
the ε-amino groups of the lysine residues. As shown in the
examples below, this is important as the cysteine side-chain
thiol group can be targeted selectively by a variety of
chemical methods, leading to disulfide bonds or more stable
thioether bonds, and even, in the special case of thiol
reacting with thiocarboxyl, an amide bond is formed by rear-
rangement.

In a practical example, peptides to be coupled were syn-
thesized with cysteine either at positions corresponding to
cysteine positions in the natural sequence or in the part of
the peptide that was preferred to be turned inward to the
backbone peptide. The following peptides were coupled in this
way to a non-dendritic peptide carrier, structure 1 (Table

1), or, in the case of IL-1, IFNγ-1, IFNγ-2, and TNFa, the
lysine side-chain formerly protected with Dde in the struc-
ture 7 non-dendritic peptide carrier of Table 1:

VQGEESNDKC (IL-1β)

CVQGEESNDK (IL-1β)

CPSTHVLLTHTI (TNFα)

HGTVIESLESLNNYFNSSGIDVEEKSLFLDIWRNWQK(C) (IFNγ-1)

AKFEVNNPQVARAAFNELIRVVHQLLPESSLRKRKRSRC (IFNγ-2)

CGMTAEDLQTRYN (PalA)

CTEADYAKNRAVLEY (PalA)

CSGGKGSFDLEDV (Tbp-2)

CPKGGNYKYIGTWD (Tbp-2)

NGSVGAVFGAK (Tbp-2)

AELGGQFHHKSENG (Tbp-2)

CASQRDRFQVHNPHENDA (SIF)

CKSQSGIEKTTRILHHANISESTQQN (SIF)

CQATAKMAEEQLTTLHVRSEQQS (SIF)

After synthesis of these peptides on a modified Rink-resin
followed by cleavage, analysis and purification, they were
coupled to a non-dendritic backbone peptide in which the ε-
amino groups were converted to thiols by reaction with SPDP
in NMP at 20 times molar surplus (resulting in a negative
Kaiser test). Peptides were coupled in 2-3 times molar sur-
plus in NMP for 2 hours at room temperature. This coupling
proceeded by the formation of disulfide bonds with a simulta-
neous release of pyridine-2-thione which can be measured
spectrophotometrically at 343 nm to indicate the degree of
substitution (molar extinction coefficient is 8080), and it
was found that the substitution was above 85% of the theor-
etically available attachment points in the non-dendritic
peptide carrier. Hereafter, the derivatized non-dendritic
peptide carrier was liberated from the resin as usual.

In another synthesis, these and other cysteine containing
peptides will be coupled to a bromoacetyl-modified backbone
peptide as thioethers. Using the reverse approach, bromoacet-
ylated peptides will also be coupled to a cysteine-derivatiz-

ed backbone peptide; in these peptides, the bromoacetyl group
will be introduced by bromoacetic anhydride (on primary
amines, typically on the α-amino group), or by using special
amino acids (BBAL (Inman 1991)) with bromoacetylated side-
chains during synthesis, to include the functionality at any
site in the chain. With the bromoacetic anhydride method,
natural peptide fragments will also be coupled, ensuring α-
amino reactivity of the bromoacetic anhydride by keeping the
pH at 6.0, performing the reaction in aqueous buffer at 0°C,
adding bromoacetic anhydride in organic solvent below 1/100
volume.

Coupling to standard backbone of Ser-substituted peptide by chemoselective ligation.

Specific N-terminal coupling of a Ser N-terminated (poly)-
peptide can be obtained after oxidation under specific condi-
tions. Such oxidation yields an N-terminal carbonyl function,
specifically reactive with acylhydrazines (hydrazides) and
with hydroxylamines, yielding hydrazones and oximes, respect-
ively.

As an example, the peptide to be coupled will be synthesized
with an N-terminal S, cleaved and purified, and oxidized in
solution by 2 mM periodate in 50 mM imidazole/HCl, pH 6.9 for
10 minutes before SepPack purification. Then the peptide will
be reacted with a hydrazide-derivatized non-dendritic back-
bone peptide carrier (in which free amino groups have been
derivatized to hydrazides by TBTU/HOBt/NMM-mediated coupling
of Boc-monohydrazide succinic acid) in solution overnight in
an aqueous buffer at a pH between 4 and 5, using two
hydrazide equivalents for each oxidised peptide to be
coupled. Reduction of the formed hydrazone to the substituted
hydrazine will be performed for two days with 0.2 M cyanobo-
rohydride at pH between 4 and 5.

Example 8

Indirect coupling: Attachment by coupling presynthesized, side-chain non-blocked peptides by C-terminal-selective chemistry

By way of chemoselective coupling, it is possible to intro-
duce presynthesized peptides on a non-dendritic peptide
carrier (NDPC) without needing to employ side-chain or α-
amino protection. In this example, the NDPC has to be deriva-
tized in order to obtain the desired selective reactivity.

In a planned first example, the peptide to be coupled will be
synthesized on a Novasyn KB-resin and then cleaved after the
final piperidine treatment followed by a TFA-treatment.
Cleavage will be performed by hydrazine in dioxane/methanol
as detailed in Example 4, to yield the peptide hydrazide,
which will then be purified by preparative reverse phase
HPLC. The NDPC will be derivatized with S (serine) on all
attachment points, periodate oxidized to yield α-oxoacyl-
groups (neutral pH in imidazole buffer, see Example 7) and
then reacted with the peptide-hydrazide at a pH between 4.5
and 5, followed by cyanoborohydride reduction of the
hydrazone to hydrazine as shown in Example 7 (all operations
performed on the solid phase attached peptide).

In another possible example, introduction of carbonyl groups
on the NDPC may be accomplished with 2,2-dimethoxyacetic
acid, being deprotected with concentrated HCl.

In yet another planned example, the peptide to be coupled may
be synthesized as its thiocarboxylic acid on a solid phase,
using the special linker of Yamashiro (Yamashiro 1988, used
by Schnölzer 1992, and Dawson 1994), 4-[(Boc-aminoacyl)thio-
benzyl]-phenoxy acetic acid, yielding the thiocarboxylic acid
upon cleavage with HF/10% p-cresol. After purification, the
peptide thiocarboxylic acid is coupled to a solid-phase bound
NDPC that is further derivatized with bromoacetic acid to

yield bromoacetyl-substituted amino groups as attachment
points. The final product is expected to contain the branch-
peptides attached as stable thioesters.

Example 9

Attachment of naturally-derived peptides

Chemical unambiguity is most easily obtained by a high degree
of chemical selectivity. This way a defined orientation and
stoechiometry is obtained. This is most easily obtained if
the natural peptide has a cysteine or a N-terminal serine
that can be used for coupling by the methods given above. If
this is not the case, peptides may be temporarily protected
and coupled as shown in Example 7. If this is not suitable,
some selectivity can be obtained, using conventional coupling
methods, that is e.g. glutaric aldehyde (coupling to amino-
and thiol groups), carbodiimides (coupling to amino groups),
and m-maleimide benzoyl-N-hydroxysuccinimide esters (coupling
from amino- to thiol-groups), by optimizing relative reactant
concentrations, pH, temperature and solvent. This may also be
combined with a purification step. Another example is the use
of haloacetic anhydride to selectively introduce e.g. bromo-
or chloroacetyl groups at the α-amino group only in a free
peptide. This group will then react with thiol groups.

In an example, this will be performed with a presynthesized
peptide as a model of a naturally occurring peptide. The
peptide will be bromoacetylated in solution in 0.1 M 2-(N-
morpholino)ethane sulfonic acid, pH 6.0, using bromoacetic
anhydride prepared from the acid by DCC-mediated activation,
in NMP, added at 1/100 (vol/vol) to the aqueous peptide
solution, and incubating for 3 x 3 minutes at room tempera-
ture (adding new reagent each time). After purification, the
peptide will be coupled to a cysteine-derivatized standard
backbone peptide until negative Ellman-test.

Example 10

Attachment of carbohydrates by coupling naturally-derived carbohydrates

In these experiments, a carbohydrate immunogen is derived
from *Salmonella typhimurium LPS* (lipopolysaccharide). The
salmonella LPS is cleaved by mild acid treatment into its
lipid part (insoluble precipitate) and its carbohydrate part
(soluble). The soluble LPS carbohydrate is obtained after
centrifugation and then oxidized by sodium metaperiodate for
10 min. at 0.1 M in the dark, followed by rapid desalting on
a Pharmacia PD-10 column. Also N-acetyl-D-galactosamine is
coupled. Both the LPS oligosaccharide and N-acetyl-D-galac-
tosamine constitute well-known carbohydrate epitopes and may
be shown by monoclonal antibodies to remain intact after
coupling as decribed below.

Coupling to non-dendritic peptide carrier

Blomberg's methodology (Blomberg 1993) is used to introduce
the carbohydrate at amino groups in a non-dendritic backbone
peptide. The oxidized carbohydrate is allowed to react in
molar excess with the solid-phase bound backbone peptide in
DMSO overnight at 60^{0}C, followed by cooling to room tempera-
ture and reaction with acetic anhydride (10 equivalents) for
4 hours at room temperature.

In another embodiment, N-acetyl-D-galactosamine is coupled by
its reducing end to the non-dendritic peptide carrier (NDPC)
to constitute a Tn-antigen that is reactive with a monoclonal
antibody against Tn-antigen.

The retention of the antigenic structure of the carbohydrate
is shown by performing an ELISA, coating with the easily
coatable carbohydrate derivatized NDPC, and detecting it with
a mouse monoclonal antibody against *Salmonella typhimurium*
LPS carbohydrate (O-chain specific antibodies) in the first

embodiment and with the monoclonal antibody directed against
Tn-antigen in the second embodiment.

Coupling to hydrazide-terminated "anchoring polymer"

In a possible example, the oxidized carbohydrate is mixed at
room temperature with the backbone peptide hydrazide (see
Example 4) at 1/1 mol/mol in 0.1 sodium acetate, pH 5.5, and
incubated for 1 hour. The resulting conjugate is easily
purifiable by Sephadex G50 gelfiltration, monitored at 280
nm. HPLC-analysis will show that it elutes before the noncon-
jugated peptide on a C-18 reverse phase column (running
conditions as decribed for Fig. 1). The retention of the
antigenic structure of the carbohydrate is shown by perform-
ing an ELISA, coating with the easily coatable lipopeptide-
carbohydrate complex, and detecting it with a mouse
monoclonal antibody against *Salmonella typhimurium* LPS carbo-
hydrate (O-chain specific antibodies).

In another example, N-acetyl-D-galactosamine is coupled by
its reducing end to the NDPC to constitute a Tn-antigen, that
is reactive with a monoclonal antibody against Tn-antigen.

Another planned example involves the use of the following
chemical strategy for attaching the same carbohydrate
antigens:

The carbohydrate is derivatized with a general amino- and
hydroxyl-reactive substance such as a carbodiimide, divinyl-
sulfone or cyanogen bromide. This is followed by reaction
with a diamine spacer such as, e.g., 1,4-butanediamine, and,
subsequently, the derivative is bromoacetylated and then
reacted with a cysteine-derivatized NDPC. In another embodi-
ment, the said derivative is reacted with SPDP before reac-
tion with the cysteine-derivatized NDPC.

Coupling to hydrazide-substituted standard-backbone

In a possible example, this is done as above, using a non-
dendritic carrier peptide derivatized with Boc-monohydrazide
succinic acid to carry hydrazide groups as attachment points.

Example 11

Attachment of a hapten

Haptens are useful as labels for model experiments as they
can be detected specifically either by commercially obtain-
able antibodies (with the haptens digoxigenin and trinitro-
phenyl) or by avidin or streptavidin (with the hapten
biotin). Biotin was easily incorporated into the non-
dendritic peptide backbone either using the succinimide ester
or activating free biotin by TBTU/HOBt/NMM in NMP before
coupling to the amino groups. Digoxigenin could also be
coupled as the succinimide. Trinitrophenylsulphonic acid
reacted rapidly with amino groups to introduce triphenyl
groups. In all cases, the reaction could be followed by the
Kaiser test.

Example 12

Attachment of DNA/RNA by coupling

DNA or RNA may be incorporated into the non-dendritic peptide
carrier or coupled as any other antigenic moiety as a branch,
part of a branch, or as an "orthogonal" branch, condensing
the aldehyde of a C-formyl nucleoside to form an imine link-
age which is reduced by reductive alkylation to form a methyl
alkylated amine bond (Vasseur 1992). Subsequently, the resul-
ting 3'-bound nucloside can be prolonged to an oligonuclotide
using automated DNA-synthesis, preferably coupling the DNA
before peptide and using the coupled DNA either directly for
encoding an interesting peptide or for binding a piece of
"natural" DNA to be expressed in the host by hybridization to
the non-dendritic peptide carrier or for direct stimulation
as with the CG oligonucleotide of Klinman (1996). In a typi-

cal application, only one DNA-copy is coupled per non-dendritic peptide carrier (NDPC).

In one further possible example, an oligonucleotide corre-sponding to a T-cell stimulatory peptide will be coupled to one specific lysine selectively deprotected side-chain using Dde or Mtt as an orthogonal protecting group to Boc. Subsequently, the rest of the lysine side-chains is deprotec-ted by TFA-treatment and antigenic peptides are coupled directly by the methods outlined above. Antigenic peptides to be used may include GEIKNCSFNISTSIRGKVQKEYAFF (gp120 peptide), and the DNA-encoded T-cell stimulatory peptide may be, e.g., QYIKANSKFIGITE (tetanus toxoid peptide). In a further synthesis, a piece of binding DNA will be incorpor-ated into the NDPC to bind the polyepitopic DNA defined by Thomson (1995) as mentioned in Example 4.

Example 13

Attachment of DNA/RNA by non-covalent inclusion

In this proposed example, a non-derivatized peptide carrier (NDPC) will be derivatized at one specific Dde-protected lysine side-chain with a polylysine chain consisting of 10 and 15 Dde-protected lysines (two different experiments), the N-terminal lysine being capped by acetic anhydride. This chain will be left in the protected state, while the rest of the backbone lysine side-chains is deprotected and used for coupling of an antigenic peptide including GEIKNCSFNISTSIRGK-VQKEYAFF (gp120 peptide). Hereafter, all side-chain protect-ing groups will be cleaved, and the peptide complex will be released, purified, and mixed in an aqueous buffer at neutral pH and with a moderate ionic strength with the appropiate DNA in a purified state or in a defined mixture with other defined DNAs. One such DNA corresponds to the tetanus toxoid T-cell peptide QYIKANSKFIGITE. Another such mixture is a mixture of DNAs each corresponding to a promiscous T-cell

epitope. Yet another DNA to be employed is the "poly-epitope" DNA of Thomson (1995) mentioned in Example 4.

Example 14

Attachment of DNA/RNA by hybridization to PNA (peptide nucleic acid)

These planned syntheses will proceed as in Example 12, using peptide nucleic acids (PNA, WO 95/01369) as building blocks on the selectively deprotected lysine side-chain defining the PNA sequence to be able to bind by hybridization the tetanus toxoid DNA corresponding to the T-cell stimulating peptide QYIKANSKFIGITE, and, in another experiment, the polyepitope DNA of Thomson (1995) mentioned in Example 4.

Example 15

Attachment of DNA/RNA by binding to peptide-bound intercalators

Another way to bind nucleic acids is to enclose in the structure an intercalator. This will be done by coupling quinoline as a thioether to a cysteine side-chain (Brown 1994) included in the C-terminal part of the non-dendritic peptide carrier. The cysteine thiol will be deprotected before Fmoc-deprotection to allow for substitution with quinoline. Hereafter, synthesis will be continued and, as the last step, lysine side-chains will be deprotected and used for coupling of an antigenic peptide including GEIKNCSFNISTSIRGKVQKEYAFF (gp120 peptide). Hereafter, all side-chain protecting groups will be cleaved, and the peptide complex will be released, purified, and mixed in an aqueous buffer at neutral pH and with a moderate ionic strength with the appropriate DNA in a purified state or in a defined mixture with other defined DNAs. One such appropriate DNA is the DNA corresponding to the tetanus toxoid T-cell peptide QYIKANSKFIGITE. Another such mixture is a mixture of DNAs each corresponding to a

promiscous T-cell epitope. Yet another DNA is the "poly-
epitope" DNA of Thomson (1995) mentioned in Example 4.

Example 15A

The use of a non-dendritic peptide carrier for coupling of antigens illustrating its use in a kit

The use of the non-dendritic peptide carrier (NDPC) in a kit
format is illustrated by the following planned example:

An NDPC will be synthesized on a RINK-MBHA-type solid phase
from Novabiochem to a substitution of from about 0.05
μmoles/g to 0.2 μmoles/g, preferably between about 0.05 to
0.1 μmoles/g. The NDPC will be included in the sequences
listed in Example 1 and will, for illustrative purposes,
include a normal NDPC without any auxiliary segments in
addition to NDPCs containing one side-chain protected auxili-
ary segment selected from a tuftsin tetramer, the
interleukin-1 nonapeptide (VQGEESNDK), and T-cell stimulatory
peptides mentioned in Example 4. After synthesis, attachment
points will be created by cleavage of side-chain protection
groups that are protected orthogonally to the other side-
chain functionalities of the NDPC (see Example 1).

In one example, the NDPC-solid-phase will then be derivatized
with SPDP, washed, dried, and stored for different time
periods until tested for the ability to bind Cys-containing
peptides as measured by the release of pyridin-2-thion and by
the ability to react with antibodies against the immobilized
peptide. Peptides to be immobilized will include the Tbp-2--
peptide of Example 5 and a biotinylated peptide which can be
detected by avidin.

Also, an NDPC derivatized with bromoacetyl groups will be
used for the attachment of the same Cys-containing peptides.

In other examples, peptides will be attached by EDC, DSS, and
by carboxylic activation with TBTU/HOBt/NMM or other equival-
ent activation procedures. Also, blocked peptides will be
attached.

In addition, the attachment of haptens, including biotin and
digoxigenin, and carbohydrates will be shown.

In all cases, the final complex will be liberated by TFA and
analysed by HPLC.

Such conjugations are expected to be performed in a quick
two-step procedure consisting in a conjugation reaction
followed by liberation and work up as usual.

Example 16

**Preparing Immune-stimulating Complexes (Iscoms) containing
derivatized non-dendritic peptide carriers**

Iscoms are easily prepared using standard methods (Morein
1984, Mowat 1992, Current Protocols in Immunology, 1992,
section IV: "Preparation of Immune Stimulating Complexes",
2.11.1-2.11.12), and lipopeptides insert themselves with a
minimal perturbation of the Iscom structure and orientating
their hydrophilic parts outwards.

A non-dendritic backbone peptide carrier will be synthesized
as above, side-chain deprotected, and used for coupling or
synthesizing side-chain antigenic peptides before cleavage.
After purification, peptides will be incorporated into Iscoms
by mixing with cholesterol, phosphatidylcholine, and quil A,
according to standard procedures referred to above.

As an illustrative example, the non-dendritic peptide carrier
palmitate-GKGKGKGKGKGG was synthesized in a 130 μmol/g scale
on a Pepsyn KB solid phase (on the base-labile 4-hydroxyme-
thyl benzoic acid linker). After completion of synthesis,

Boc-protection groups on the K-residues were removed by a 2 x
15 minutes wash in 95% TFA/water with a concurrent change
from negative to a positive Kaiser test. After neutralization
and wash in NMP, biotin was attached by TBTU/HOBt/NMM, using
a 2 times surplus of biotin and 2 couplings of 30 minutes at
room temperature preceeded by 10 minutes of preactivation.
After washing in NMP and water, the side-chain biotinylated
peptide was cleaved by NaOH/water as in Example 1, worked up
by desalting on preparative HPLC followed by freeze-drying,
and then used without further purification for inclusion into
Iscoms by mixing the peptide with the Iscom-forming reagents
(peptide/quil A = 1/5, w/w) following standard procedures
(see above). Iscoms were characterized by sucrose-gradient
ultracentrifugation, and fractions were analysed by electron
microscopy for Iscom-formation and by HRP-streptavidin ELISA
for biotin. The Iscom-containing fractions showing well-
defined normal Iscom-structures by electron microscopy were
found by the ELISA to contain also the biotin-peptide in a
presentation where the biotin was accessible to the reaction
with the HRP-streptavidin-reagent. The purified Iscom-frac-
tion retained avidin-reactivity even after extensive dia-
lysis, which was easily shown by dot-blotting. Furthermore,
peptides were demonstrated in the purified Iscoms by direct
reverse-phase HPLC analysis of the Iscoms.

Example 17

**Preparing and using loaded Iscoms for attachment of antigenic
moieties.**

Carrier with amino groups

In one embodiment, the non-dendritic peptide carrier (NDPC)
lipopeptide of the invention is included in an Iscom before
coupling antigenic branch-moieties. This allows the prepara-
tion of ready-made "loaded" Iscoms, ready for en-bloc coup-
ling of branch-moieties in a manner which is special by

employing a synthetic and specifically designed NDPC lipopep-
tide as the Iscom-inserted carrier.

A standard backbone-lipopeptide will be synthesized by the
methods given above, deprotected, cleaved from the solid
phase, purified, lyophilized and solubilized, and incubated
together with Iscom-forming substances according to Morein
(1984). In a series of incubations, the peptide-to-quil A
ratio will be varied around 1:5, such as from 1:2 to 1:10.
After preparation of Iscoms, they will be analysed for inte-
grity and general appearance by electron microscopy, for
accessibility of backbone-peptide amino groups by hapten-
derivatization of free amino groups (using gold-labelled
immunoglobulin indirectly binding to trinitrophenyl groups
introduced on the amino groups, or by gold-labelled avidin
labelling biotinylated amino groups) followed by inspection
by electron microscopy and for loading "density" (amount of
peptide pr. quil A) by quantitative HPLC of peptide-loaded
Iscoms.

In one particular embodiment, the NDPC will be synthesized on
a solid phase using a water-cleavable linker (see Hoffmann,
1994). Then the peptide is expected to be obtained in its
Iscom-enclosed form simply by mixing the finished, but still
solid-phase bound peptide with Iscom-forming agents in aque-
ous buffer for between 2-24 hours, then filtering off the
solid-phase.

Compatible coupling schemes for the subsequent coupling of
antigenic branch structure include methods that do not
involve the use of organic solvents and/or extremes of pH
(e.g. Regenmortel 1988). As examples, peptides included in
the list in Example 5 will be coupled using such coupling
schemes. The resulting peptide-loaded Iscoms will be analysed
by electron microscopy and by HPLC for loading "density".

Carrier with a C-terminal hydrazide for coupling oligosaccha-
rides

A backbone-peptide containing a C-terminal hydrazide, prefer-
ably obtained by cleaving the peptide from the solid-phase by
hydrazine/NMP as detailed above (Novabiochem 1994), will be
included as such in an Iscom, simply by mixing the components
in PBS and incubating at room temperature for 24 hours, then
purifying Iscoms by sucrose gradient-centrifugation. The
carbohydrate moiety will be either N-acetyl galactosamine or
natural or oxidized Salmonella LPS O-carbohydrate (see
Example 10) and will be attached as detailed in Example 10.
Iscom will be inspected by electron microscopy and further
analysed by studying the binding to the Iscoms of a number of
carbohydrate-specific antibodies, including anti-Tn anti-
bodies and anti-LPS antibodies both by gold-labelled anti-
bodies in electron microscopy and by ELISA using the loaded
Iscoms for coating.

Example 18

**Application of a non-dendritic peptide carrier for immuniz-
ation to create protection against virus: The mink enteritis
virus model**

In this model, the virus capsid peptide, DGAVQPDGGQPAVRNER,
originally derived from canine parvovirus constitutes a
protective epitope (Langeveld 1995) against mink enteritis
virus induced disease.

In the example, this peptide will be coupled in the "C-out-
ward" orientation by including a N-terminal cysteine and
coupling to a SPDP-derivatized non-dendritic peptide carrier.
"N-outward" orientation will be achieved by normal sequential
synthesis on the same type of backbone peptide.

As the non-dendritic backbone peptide carrier, the backbone peptide no. 6 of Table 1 will be used as well as the same peptide extended C-terminally with either

-a tetanus toxoid T-cell stimulatory peptide, TT (QYIKANSKFI-GITE),
-the IL-1ß nonapeptide (VQGEESNDK), or

-a tuftsin tetramer ([TKPR]$_4$)
using Mtt-protected side-chains in attachment point-lysine residues and Boc-protection for other lysine residues.

After synthesis of peptides corresponding to these four groups and HPLC and MS analyses, immunization will be performed by one subcutaneous injection of 50-200 μg peptide-construct in 1000 μl PBS in the following groups of animals:

Backbone [C-outward]$_4$
Backbone [N-outward]$_4$
Backbone [C-outward]$_4$ + Alhydrogel
Backbone [N-outward]$_4$ + Alhydrogel
Backbone-TT peptide [C-outward]$_4$
Backbone-TT peptide [N-outward]$_4$
Backbone-tuftsin [C-outward]$_4$
Backbone-tuftsin [N-outward]$_4$
Backbone-IL-1 [C-outward]$_4$
Backbone-IL-1 [N-outward]$_4$
(10 groups of animals, 4 in each group)

Immunizations will be followed by whole virus challenge at day 21, and animals will be checked for infection (isolation of virus) and clinical disease for 14 days after challenge. Titers will be followed throughout by ELISA employing the relevant immunization peptide as the coating antigen.

Example 19

Non-dendritic peptide carriers for use as diagnostics in the diagnosing of infectious diseases

The use of the non-dendritic peptide carrier in a diagnostic immunoassay is illustrated by the following example in which peptide sequences derived from an infectious agent are being recognized by antibodies present in samples from humans exposed to the infectious agent.

Diagnostic immunoassay for detection of HIV-1 and HIV-2 infection

Test-serum panels

Test serum panels from Boston Biomedica, Inc. are used throughout the diagnostic testing.

Preparation of non-dendritic peptide carriers carrying peptides specific for HIV-1 and HIV-2

The following peptide sequence specific for HIV-1 and often recognized by HIV-1 infected patients is selected for HIV-1:

gp41 (aa598-609):

Leu-Gly-Ile-Trp-Gly-Cys-Ser-Gly-Lys-Leu-Ile-Cys

The following peptide sequence specific for HIV-2 and often recognized by HIV-2 infected patie..rs is selected for HIV-2:

gp36 (aa587-605):

Leu-Gln-Asp-Gln-Ala-Arg-Leu-Asn-Ser-Trp-Gly-Cys-Ala-Phe-Arg-Gln-Val-Cys-His-Thr

Different diagnostic components are produced by coupling
peptide sequences to non-dendritic peptide carriers following
the procedure described in Examples 1 and 2.

The non-dendritic peptide carrier provides a multimer presen-
tation of each of the peptide sequences which can then be
used in a variety of diagnostic assays, including ELISA,
line-blotting, and agglutination assays.

Each non-dendritic peptide carrier carrying the HIV specific
peptides is tested in parallel with the same peptide not
linked to the carrier peptide and with the corresponding
recombinant protein HIV-1 gp41.

ELISA-assay

Synthetic peptides were coated on Maxisorp microtitre plates
(Nunc, Roskilde, Denmark). Peptides (10 μg/ml) were coated to
the plates in 100 mM $NaHCO_3$ at pH 9.6 or in 0.1 mM glycin-HCl
at pH 2.5. All coatings were performed overnight at 4°C. The
wells were washed 4 times in 0.5 M NaCl, 3mM KCl, 1mM KH_2PO_4,
8mM $Na_2HPO_4,2H_2O$, 1% Triton X-100. This washing procedure was
repeated after each of the following incubation steps:

1)   Plasma samples, 1% (v/v) in incubation buffer (washing
     buffer plus 15 mM bovine albumin, pH 7.2), were incubated
     for 1 hour at room temperature.

2)   100 μl per well of horse radish peroxidase-conjugated
     rabbit anti-human class-specific antibodies (DAKO,
     Copenhagen, Denmark) diluted in incubation buffer, was
     added at room temperature for 1 hour.

Enzyme activities were quantitated after addition of 100 μl
per well of 0.67 mg/ml 1,2-phenyldiamine hydrochloride (DAKO)
dissolved in 100 mM citric acid-phosphate buffer, pH 5.0
containing 0.015% (v/v) H_2O_2. The reactions were stopped by

adding 50 μl per well of 2.5 M H_2SO_4, and the optical den-
sities were measured in an ELISA scanner at 492 nm.

All tests were done in duplicate.

<u>Result</u>

The ELISA procedure was optimized by testing different con-
centrations of the peptides alone and coupled to the car-
riers, respectively. Fig. 5 shows antibody reactivity of a
HIV-1 seropositive donor against dilution curves of the gp41
(aa598-609) peptide, being tested alone and linked to the
carrier. The ELISA procedure is optimized by testing diffe-
rent concentrations of the peptides.

Fig. 6 shows dilution curves of sera obtained from HIV-2
seropositive donors and HIV-2 seronegative donors tested
against the non-dendritic peptide carrier derivatized with
gp36 (aa 587-605) peptide. Positive signals were obtained at
all dilutions of HIV-2 seropositive sera tested while the
seronegative control sera were negative in all the dilutions
tested.

Fig. 7 shows the results with fixed dilutions of sera
obtained from a panel of HIV-2 and HIV-2 seronegative donors
tested against gp36 peptide alone and coupled to the non-
dendritic peptide carrier. The sera were also tested against
recombinant protein HIV-2 gp36. All the seropositive donor
sera were reactive in the assay using gp36 peptide coupled to
the non-dendritic peptide carrier, while no reactivity was
detectable with the seronegative control sera.

Fig. 8 shows dilution curves of sera obtained from one HIV-2
seropositive donor and one HIV-2 seronegative donor tested
against the gp36 peptide alone and coupled to the non-
dendritic peptide carrier. The sera were also tested against
recombinant HIV-2 gp36. The peptide coupled to the carrier

was recognized at higher dilutions of the seropositive serum
compared to the peptide alone.

The HIV-2 specific gp36 peptide coupled to the non-dendritic
peptide carrier without any modifications was as useful for
diagnostic purposes as was the whole recombinant HIV-2 gp36
protein. The gp36 peptide coupled to the non-dendritic car-
rier was recognized by HIV-2 seropositive sera at lower
dilutions than the peptide alone.

Dot-blotting assays for diagnosing for HIV-1 and HIV-2:

<u>Procedure</u>

The non-dendritic peptide carrier derivatized with HIV pepti-
des (app. 50 μg/ml) as well as the peptide alone is coated on
nitrocellulose membranes in 2.5 μl volumes. Blocking of
nitrocellulose is performed for 10 min with 0.05 M Tris
buffer, pH 7.4 + 0.5 M NaCl + 0.5% Tween 20. Test-sera are
incubated for one hour diluted in assay-buffer: Tris buffer,
pH 7.4 + 0.5 M NaCl + 0.05% Tween 20. After washing, the
nitrocellulose membrane is incubated two times with assay-
buffer, incubation with peroxidase-conjugated anti-human
immunoglobulin follows. Human immunoglobulin reactivities
with peptides are detected using the substrate 3-amino-9-
ethylcarbazole. The test results are inspected visually.

<u>1st Screening</u>

First screening is performed with a panel of sera from app.
10 HIV-1 patients, 5 HIV-2 patients, and 50 uninfected indi-
viduals.

Sensitivity and specificity of the assay are estimated, and
the reproducibility of the assay is tested by repeated tests
of the serum samples.

<u>2nd Screening</u>

Second screening is performed using 14 sera with low titers
of antibodies against HIV-1 virus. This test using sera with
low or uncertain reactivities against HIV allows for evalu-
ation of the sensitivity and specificity of the non-dendritic
peptides in detecting antibodies against HIV infection as
compared to that of other commercial assays.

3rd Screening

Third screening is performed using sera from app. 5 donors
from which several blood samples have been collected around
the time of sero conversion following infection. This test
estimates the size of time-window from exposure to sero
conversion at which point people start producing HIV-1-speci-
fic antibodies

Example 20

Non-dendritic peptide carriers derivatized with immunogenic peptides for use as vaccines

The non-dendritic peptide carrier derivatized with
immunogenic peptides can be used for immunization purposes to
stimulate antibody and T-cell responses against any
immunogenic agent. The non-dendritic peptide carrier can thus
be used for vaccination purposes to protect against
pathogenic microorganisms. One example of this use of the
non-dendritic peptide carrier is given below where mice are
immunized against the parasitic disease malaria.

**Immunization of mice with a non-dendritic peptide carrier
derivatized with a peptide sequence from the erythrocyte
binding antigen-175 from *Plasmodium falciparum***

Materials and methods

Mice were immunized with a non-dendritic peptide carrier derivatized with the synthetic peptide:

TLTKEYEDIVLKSHMNRESDD

This peptide is derived from the malaria parasite *Plasmodium falciparum*. The peptide covers a sequence of the erythrocyte binding antigen-175 (EBA-175) involved in the parasite invasion of erythrocytes. However, the sequence is normally not recognized by the immune system during infections.

Experimental design

Female 6-8 weeks old (C57BlxBALB/c)F1 mice were used in these studies.

BCG primed mice were immunized intraperitoneally 3 times, day 0, 21, and 49, with 16 μg of the non-dendritic peptide carrier derivatized with EBA-175 peptide conjugated to Purified Protein Derivative (PPD) with and without absorption to aluminium hydroxide.

In the second set of experiments, mice were immunized with the non-dendritic peptide carrier derivatized with the EBA-175 peptide without any conjugations. Some mice were immunized intraperitoneally 3 times, day 0, 21, and 49, with 16 μg of the derivatized non-dendritic peptide carrier, while some other mice were immunized subcutaneously 3 times, day 0, 21, and 49, with 16 μg of the derivatized non-dendritic peptide carrier mixed 1+1 with Freund's complete (1st immunization) or incomplete (2nd and 3rd immunization) adjuvant.

The mice were bled on days -1, 12, 33, and 61. Sera were collected from the bleedings and tested in ELISA for antibody reactivity against the peptide used for immunization.

Serum antibody reactivity with synthetic peptides in ELISA

The synthetic peptide conjugated to ovalbumin (1 μg/ml) was
coated in 100 mM NaHCO-3, pH 9.6 on Maxisorp microtitre
plates (Nunc, Roskilde, Denmark). All coatings were performed
overnight at 4°C. The wells were washed 4 times in 0.5 M
NaCl, 3mM KCl, 1mM KH_2PO_4, 8mM Na_2HPO_4, 1% Triton X-100. This
washing procedure was repeated after each of the following
incubation steps:

1)   Mice sera made to 1% (v/v) in incubation buffer (washing
      buffer plus 15 mM bovine albumin, pH 7.2) were incubated
      1 hour at room temperature,

2)   100 μl per well of biotinylated rabbit anti-mice IgG
      antibodies (Amersham) diluted in incubation buffer, and

3)   100 μl per well of streptavidin peroxidase (DAKO,
      Copenhagen, Denmark) diluted in incubation buffer was
      added at room temperature for 1 hour.

Enzyme activities were quantitated after addition of 100 μl
per well of 0.67 mg/ml 1,2-phenyldiamine hydrochloride (DAKO)
dissolved in 100 mM citric acid-phosphate buffer, pH 5.0
containing 0.015% (v/v) H_2O_2. The reactions were stopped by
adding 50 μl per well of 2.5 M H_2SO_4, and the optical den-
sities were measured in an ELISA scanner at 492 nm.

On each ELISA microtitre plate, one positive control serum
and one negative control serum were assayed as well as con-
trol wells without serum (background). All tests were done in
duplicate.

Results

Fig. 9 shows that mice produced antibodies in response to
immunization with the non-dendritic peptide carrier derivati-
zed with EBA-175 peptide-PPD conjugate. The strongest anti-

body response was detectable after 3 immunizations. Absorption to aluminium hydroxide enhanced antibody production after one immunization but not after two or three immunizations.

Fig. 10 shows that mice produced peptide-specific antibodies in response to subcutaneous immunization with the non-dendritic peptide carrier derivatized with EBA-175 peptide mixed with Freund's adjuvant. The response was detectable after two immunizations. Some mice showed a weak peptide-specific antibody reactivity in response to the non-dendritic branched peptide alone after 3 immunizations intraperitoneally.

IgG1 reactivity is a marker of Th2 reactivity, while IgG2a reactivity is a marker of Th1 reactivity.

Mice immunized with the non-dendritic peptide carrier derivatized with EBA-175 peptide and Freund's adjuvant showed both IgG1 and IgG2a reactivity to the peptide (Fig. 14 and 15).

Immunization with two different non-dendritic peptide carriers each derivatized with a peptide sequence from HIV-1

Mice were immunized with a non-dendritic peptide carrier derivatized with the peptide aa 152-176 from HIV-1 gp120 having the amino acid sequence:

GEIKNCSFNISTSIRGKVQKEYAFF

This peptide has known B- and T-cell epitopes.

Another peptide for use as a vaccine linked to the non-dendritic peptide carrier is the peptide from HIV-1 gp41 comprising the following amino acid sequence:

LERLLL

This peptide has interleukin-2 homology (Reiher III, 1986).

Experimental design

Female 6-8 weeks old mice, BALB/cJ, were used in these studies.

One group of mice was immunized intraperitoneally 3 times, day 0, 21, and 49, with 16 μg of the non-dendritic peptide carrier derivatized with the HIV-1 peptides, while another group of mice was immunized subcutaneously 3 times, day 0, 21, and 49, with 16 μg of the non-dendritic peptide carrier derivatized with the HIV-1 peptides mixed 1+1 with Freund's complete (1st immunization) or incomplete (2nd and 3rd immunization) adjuvant.

The mice were bled on days -1, 12, 33, and 61. Sera were collected from the bleedings and tested by ELISA for antibody reactivity against the peptide, using ELISA assays as described for the malaria peptide.

Results

Fig. 11 shows that mice responded to the gp120 peptide after subcutaneous immunization with the derivatized non-dendritic peptide carrier mixed with Freund's adjuvant. The strongest antibody response was detectable after 3 immunizations.

The mice responded weakly to the gp41 peptide after intraperitoneal immunization with the non-dendritic peptide carrier derivatized with the gp41 peptide alone (Fig. 16). Some mice received two non-dendritic peptide carriers derivatized with gp-120 and gp-41 peptides in combination. These mice produced GP-41 specific antibodies (Fig. 16).

Mice also showed reactivity to the recombinant proteins gp120 and gp41 in response to subcutaneous immunization with the non-dendritic peptide carrier derivatized with gp-120 peptide

mixed with Freund's adjuvant respectively two non-dendritic peptide carriers derivatized with gp-120 peptide and gp-41 peptide mixed with Freund's adjuvant (Fig. 17 and 18).

Mice produced gp-120 peptide-specific IgG1 and IgG2a antibodies in response to subcutaneous immunization with the non-dendritic peptide carrier derivatized with gp120 peptide mixed with Freund's adjuvant, but the IgG2a responses were eliminated if the non-dendritic peptide carrier derivatized with gp120 peptide was mixed with the non-dendritic peptide carrier derivatized with gp41 peptide during the immunizations (Fig. 19 and 20).

Mice produced gp-41 peptide-specific IgG1 and IgG2a antibodies in response to subcutaneous immunization with a mixture of two non-dendritic peptide carriers derivatized with the gp-41 peptide and the gp-120 peptide mixed with Freund's adjuvant, while no response was detectable after subcutaneous immunization with the non-dendritic peptide carrier derivatized with the gp-41 peptide mixed with Freund's adjuvant alone (Fig. 21 and 22). The gp-120 peptide functioned as a carrier for the gp41 peptide providing T-cell stimulation to induce IgG reactivity to the gp41 peptide.

Other vaccine constructions

Repetition of the amino acid sequences

To evaluate the importance of repeating the amino acid sequence in the immunogenic peptide, the peptides $(VTEEI)_1$, $(VTEEI)_2$ and $(VTEEI)_3$ will be tested following the same procedure as described for the other malaria peptides.

Inclusion of B-cell and T-cell epitopes

The immunogenicity of the peptide construct may be further improved in several ways such as by the inclusion of one or more B-cell and T-cell epitopes as peptides in the non-

dendritic peptide carriers in addition to the molecule against which immunity is desired.

Inclusion of universal T-cell epitopes may have the effect of overcoming MHC restriction. This aspect will be tested by linking peptides from T-cell epitopes to the non-dendritic peptide carrier in combination with a peptide against which immunization is desired. Combinations of immunogenic peptides and peptides having T-cell epitope function will be used both based on the use of the peptides:

QYIKANSKFIGITEL

and

FNNFTVSFWLHRVKVSASHLE

Both of these sequences are known to be universal T-cell epitopes (see Wang, 1995) and will be used in combination with:

(QGPGAP)$_4$ (malaria specific)

and with the TCTKEYEDIVLKSHMNRESDD

(from the erythrocyte binding antigen-175).

Testing of the peptides will be performed using the experimental design described above under Example 20.

Other interesting epitopes

Likewise, a number of other interesting epitopes may be included in the non-dendritic peptide carrier alone or in combinations. Such interesting epitopes include Tcyt epitopes for stimulation of cytotoxic T-cells, lipids or tuftsin sequence for activation of the immune system, and cytokines or bioactive cytokine sequences.

Recombinant or native immunomodulators such as cytokines may be inserted into Iscoms together with non-dendritic peptide carrier constructs. The cytokines may include e.g. interleukin 1-18, interferon-gamma, interferon-alpha, interferon-beta, and TNF (tumour necrosis factor). Alternatively, bioactive cytokine-specific amino acid sequences may be part of the sequence included in the branched peptide-construct.

A number of other HIV-1 peptides can also be used following the experiment described above. Such peptides include various other peptide sequences from gp120 and gp41.

Example 21

Non-dendritic peptide carriers for use as therapeutic agent in therapy of various diseases

Non-dendritic peptide carriers derivatized with peptide sequences capable of preventing or curing diseases or diminishing disease manifestations constitute a very interesting aspect of the invention.

To illustrate this application of the invention, an example is given below:

Non-dendritic peptide carriers for treatment of sepsis-like disease

Sepsis-like disease manifestations are induced by phospholipids from microorganisms like lipid A of gram-negative bacteria and phosphatidylinositol containing structures of malaria parasites. Beta-2-glycoprotein I is a serumprotein known to bind in general to negatively charged phospholipids. By linking sequences of this glycoprotein to the non-dendritic peptide carrier, binding to phospholipids can be mediated, whereby specific targeting of any non-dendritic

peptide carrier derivatized with a disease preventing or curring agent can be achieved.

The following peptides were synthesized:

beta-2-glycoprotein I peptide aa268-278:

KNGMLKGDKVS

This peptide binds antibodies against phospholipids and is, therefore, also relevant in connection with autoimmune diseases,

beta-2-glycoprotein I peptide aa 281-288:

CKNKEKKC

This peptide binds phospholipids.

Peptide alone and the non-dendritic peptide carrier derivatized with peptide were tested in 3 *in vivo* assays for their protective effect against sepsis-like disease manifestations. The testing involves measurement of TNF (tumour necrosis factor) levels.

Experimental design

Treatment scheme

8-12 weeks old (C57BLxBALB/c)F1 mice grouped with 2 mice in each group were used.

The peptides were mixed with the stimulating toxins (10 μg lipopolysaccharide), and the mixture was injected i.p. into the mice.

For TNF assays, priming of mice with BCG was performed 7-10 days before injection of stimulating toxins.

<u>Blood collection</u>

10 μl tail-blood were collected as EDTA plasma, and the following bleedings were performed for the TNF assay:

0, 1, 2, and 4 hours after.

<u>Cell viability</u>

Cell viability was evaluated by tryphaneblue exclusion.

<u>TNF ELISA assays</u>

Murine TNF-alpha levels in sera were measured by a commercial ELISA method performing according to the instructions of the manufacturer (Genzyme, Cambridge, MA).

Results

The non-dendritic peptide carriers decrease lipopolysaccharide toxicity in mice as measured by tumour necrosis factor concentrations in murine sera.

The non-dendritic peptide carrier derivatized with CKNKEKKC peptide or the KNGMLKGDKVS peptide inhibited TNF secretion *in vivo* in mice induced by lipopolysaccharides, while the control peptide alone caused no blocking (Fig. 23).

Other diseases

Other therapeutical uses of the non-dendritic peptide carriers according to the present invention include linking of HIV-1 protease inhibitor peptides to the construct or the linking of blood-brain barrier specific peptides such as E-selectin having the amino acid sequence:

THLVAIQNKEEIEYL

or the pertussis toxin, S_2, having the amino acid sequence:

RALTVAELRGSGDLQEYL

See Example 28.

Example 22

Inhibition of malaria antigen induced cytokine secretion by linking a beta-2-glycoprotein I peptide to a non-dendritic peptide carrier

Background

Pathogenicity of *P.falciparum* malaria parasites appears to be associated with the parasites multiplication, cytoadherence to endothelial cells, and their induction of cytokines like TNF-alpha and IL-6. Malaria drugs are designed to block parasite multiplication, but their ability to interfere with parasite cytoadherence or parasite induction of cytokine secretion is rarely investigated although a recent study showed that chloroquine decreased TNF-alpha secretion from human monocytes stimulated by lipopolysaccharide. African children may die even after they have had their parasites eliminated by malarial drugs probably because of inflammatory reactions in the brain. Drug-mediated interference with parasite cytoadherence or parasite induced production of cytokines is likely to reduce mortality and morbidity caused by malaria parasites.

Materials and Methods

Synthetic peptides

beta-2-glycoprotein I peptide aa 281-288:

KNGMLKGDKVS

This peptide binds antibodies against phospholipids and is therefore also relevant in connection with autoimmune diseases.

beta-2-glycoprotein I peptide aa 281-288:

CKNKEKKC

This peptide binds phospholipids.

Malaria parasite cultures

The *P.falciparum* isolate 3D7 was kept in continuous cultures using RPMI 1640 supplemented with 21 mM sodium bicarbonate, 25mM HEPES buffer, and 10% human serum. The parasites were grown in 4% v/v group O positive human erythrocytes.

Exoantigens of *P.falciparum* for stimulation of cytokine production

Exoantigens were affinity purified from culture medium essentially as described previously (Jakobsen et al., 1988), using as a ligand a pool of IgG from clinically immune African adults. Before chromatography, the culture medium was centrifuged at 7000 g for 10 min, filtered through a 0.22 μm membrane, and dialysed overnight at 4°C against column buffer.

Stimulation of cytokine-release from human mononuclear cells

Human peripheral blood mononuclear cells from different Danish donors were suspended in 3% (v/v) human serum in RPMI 1640 and adjusted to $2X10^6$ cells per ml; subsequently, 0.1 ml volumes were dispensed into wells of 96-well microtiter plates. Peptides in two-fold dilutions (0-100 μg/ml) and optimal concentrations of stimulating malaria antigens, diluted in RPMI 1640 with 3% serum, were then added to a total volume of 0.2 ml. per well. Mononuclear cells incubated with stimulating antigens without peptides, and cells incu-

bated with medium alone served as positive and negative
controls, respectively. The cultures were incubated overnight
for IL-6 and TNF assays.

ELISA for IL-6

Supernatants were collected and assayed for IL-6 by ELISA
procedures (Hansen et al., 1991; Jakobsen et al., 1993) with
the following modifications. ELISA Maxisorp plates (NUNC,
Roskilde, Denmark) were coated for 24 hours at 4°C with 100
μl per well of 2.5 μg/ml rabbit polyclonal IgG to human
recombinant IL-6 in 100 mM NaHCO$_3$, pH 9.6. Non-attached sites
were blocked for 1 hour by 100 ul per well of 2% human serum
albumin in phosphate buffered saline, pH 7.2 (1 hour at
37°C).

The wells were washed 4 times in 2.5% NaCl, 0.1% Tween 20
(Merck, Darmstadt, Germany). This washing procedure was done
after each of the following incubation steps:

1)   100 μl culture supernatants made to 50% (v/v) in incuba-
      tion buffer, (4% (v/v) normal rabbit serum, (DAKO code
      X902), 1% polyethylene glycol (mol.wt 6000), 2.5% NaCl,
      0.2% Tween 20 in phosphate buffered saline) were incu-
      bated for 2 hours at 37°C, dilutions of recombinant
      cytokine standards in the same incubation buffer were
      assayed in parallel with the tested supernatants.

2)   100 μl per well of biotinylated rabbit antibodies to
      recombinant IL-6 (1.5 μg/ml in 0.5% human serum albumin,
      0.1% Tween 20 in PBS) were added at 37°C for 1½ hours.

100 μl per well of 0.67 mg/ml 1,2-phenyldiamine hydrochloride
(DAKO) dissolved in 100 mM citric acid-phosphate buffer, pH
5.0, containing 0.015% (v/v) H$_2$O$_2$. The reactions were stopped
by adding 50 μl per well of 2.5 M H$_2$SO$_4$, and the optical
densities were measured in an ELISA scanner at 490 nm against
a test reference at 620 nm.

Results

Non-dendritic peptide carriers inhibited malaria parasite toxin activity *in vitro* as measured by secretion of tumour necrosis factor and interleukin-6. The peptides did not affect lipopolysaccharide toxity *in vitro*. The inhibition of malaria parasite toxin activity was not caused by toxity to the cells as cell viability was not affected.

In pilot experiments, the non-dendritic peptide carrier derivatized with the CKNKEKKC peptide inhibited IL-6 secretion induced by malaria antigens, while the control peptide alone caused only minor blocking of IL-6 secretion (Fig. 12). Means of two experiments are shown in the figure.

The non-dendritic peptide carrier derivatized with the CKNKE-KKC peptide inhibited both TNF-alpha and IL-6 secretion from human mononuclear cells stimulated with malaria parasite exoantigens (Table 3 below). The control peptide showed no blocking effect. None of the peptides blocked lipopolysaccharide induced cytokine secretion (Table 4 below). In addition, none of the peptides affected cell viability (Table 5 below), indicating that the peptide constructs have no or low toxity.

Table 3

Percentage inhibition of malaria exoantigen induced TNF-alpha
and IL-6 secretion from human peripheral mononuclear cells *in
vitro* by a derivatized non-dendritic peptide carrier (NDPC)
derivatized with peptide CKNKEKKC or KNGMLKGDKVS, and by a
control peptide-CKNKEKKC. Means and standard deviations are
shown.

Table 3		
Peptide (µg/ml)	TNF-alpha	IL-6
CKNKEKKC (NDPC) 200 100 50 25	n=7 61.9% (28.6) 47.5% (27.4) 50.0% (36.5) 34.2% (27.7)	n=7 55.5% (33.2) 34.4% (41.2) 40.8% (38.8) 24.8% (37.0)
CKNKEKKC (control) 200 100 50 25	n=7 19.6% (15.7) 15.7% (10.3) 10.7% (36.5) 12.2% (18.2)	n=7 0.7% (9.4) -6.2% (6.7) -2.4% (11.4) -3.7% (9.4)
KNGMLKGDKVS (NDPC) 200 100 50 25	n=7 13.9% (19.9) -8,9% (27.0) 14.0% (25.0) 8 5% (19.4)	n=7 19.9% (18.8) 5.3% (6.4) 1.7% (15.2) -7.6% (7.4)

Table 4

Percentage inhibition of *E.coli* lipopolysaccharide induced
TNF-alpha and IL-6 secretion from human peripheral
mononuclear cells *in vitro* by a derivatized non-dendritic
peptide carrier (NDPC) derivatized with peptide CKNKEKKC or
KNGMLKGDKVS, and by a control peptide-CKNKEKKC. Means and
standard deviations are shown.

Table 4		
Peptide (μg/ml)	**TNF-alpha**	**IL-6**
CKNKEKKC (NDPC) 200 100 50 25	n=4 -7.7% (17.5) -15.0% (25.1) -27.0% (21.7) -14.4% (41.7)	n=4 -13.0% (6.1) -13.0% (3.1) -22.5% (7.2) -12.9% (4.4)
CKNKEKKC (control) 200 100 50 25	n=4 -5.6% (7.7) 3.9% (22.7) 9.1% (7.5) 10.9% (20.0)	n=4 -6.6% (7.1) -9.5% (10.2) -4.7% (2.6) -2.7% (2.7)
KNGMLKGDKVS (NDPC) 200 100 50 25	n=4 16.4% (34.3) 2.6% (22.9) 9.5% (50.6) -3.7% (42.5)	n=4 2.0% (8.6) -4.9% (6.4) -8.5% (7.9) -7.7% (7.4)

Table 5

Percentage mononuclear cell viability after incubation with
peptides and malaria parasite exoantigens. Means and standard
deviations of 3 experiments are shown. Cell viability after
antigen stimulation without peptides was 96.3% (1.04), and
cell viability after no stimulation was 96.9% (2.95).

Table 5		
CKNKEKKC (NDPC)	**With antigen**	**Without antigen**
200 μg/ml	96.3% (1.99)	96.4% (1.89)
100 μg/ml	97.1% (1.45)	97.8% (1.43)
50 μg/ml	94.6% (1.01)	96.7% (0.71)
25 μg/ml	94.5% (3.26)	96.9% (3.26)
CKNKEKKC (control)	**With antigen**	**Without antigen**
200 μg/ml	98.0% (1.45)	96.5% (0,72)
100 μg/ml	96.8% (1.81)	97.3% (0,97)
50 μg/ml	97.2% (1.91)	96.0% (2.50)
25 μg/ml	97.5% (1.67)	98.2% (1.78)
KNGMLKGDKVS (NDPC)	**With antigen**	**Without antigen**
200 μg/ml	96.4% (2.76)	95.6% (3.01)
100 μg/ml	97.4% (1.85)	96.9% (1.00)
50 μg/ml	96.7% (1.98)	95.2% (2.29)
25 μg/ml	97.4% (1.44)	96.1% (1.75)

Example 23

Down regulation of the immune system in connection with autoimmune diseases by the non-dendritic peptide carriers

The non-dendritic peptide carrier may include immunogenic peptides in combination with bioactive peptides for specific downregulation of immune responses against any immunogenic agent. Alternatively, the non-dendritic peptide carrier derivatized with immunogenic peptides can be used in combination with immunosuppressive cytokines, like IL-10 and TGF-beta, for specific downregulation of immune responses against any immunogenic agent. Thus, tolerance to a specific peptide or protein fragment may be induced. This strategy may be used for protection against or therapy for toxic diseases, auto-immune diseases (diabetes, arthritis, sclerosis etc.).

One example of the modulating effect is given below. Mice were immunized with the non-dendritic peptide carrier derivatized with the malaria EBA-175 peptide three times, inducing high antibody reactivities against the peptides. The non-dendritic peptide carrier derivatized with the EBA-175 peptide was then mixed with murine IL-10, and immunization was performed the fourth time, followed by bleeding and screening for peptide specific antibodies.

Materials and methods

Mice were immunized with non-dendritic peptide carrier constructs containing one model-peptide:

TLTKEYEDIVLKSHMNRESDD

This peptide is derived from the malaria parasite *Plasmodium falciparum*. The peptide covers a sequence of the erythrocyte binding antigen-175 (EBA-175), involved in the parasite invasion of erythrocytes. However, the sequence is normally not recognized by the immune system during infections.

Female 6-8 weeks old (C57BIxBALB/c)F1 mice were used in these studies.

Mice were immunized with the non-dendritic peptide carrier derivatized with EBA-175 peptide. Some mice were immunized subcutaneously 3 times, day 0, 21, and 49, with 16 µg of peptide constructs mixed 1+1 with Freund's complete (1st immunization) or incomplete (2nd and 3rd immunization) adjuvant. Mice were immunized a fourth time, day 70, subcutaneously with the peptide-construct mixed with Freund's incomplete adjuvant with and without 1 µg of murine recombinant IL-10.

The mice were bled on days -1, 12, 33, 61, and 82. Sera were collected from the bleedings and tested in ELISA for antibody reactivity against the control EBA-175 peptide.

The ELISA was performed as described in Example 20 in the patent application.

Results:

Eight mice responding to EBA-175 peptide with Freund's complete adjuvant were immunized one more time (4th immunization). Four mice received non-dendritic peptide carriers derivatized with EBA-175 peptides plus Freund's incomplete adjuvant, and four mice received the same plus murine recombinant IL-10, a downregulator of the immune system.

Fig. 24 shows that mice immunized with the peptide-adjuvant combination alone had a stable or slight decline in antibody reactivity, while mice immunized with the peptide-adjuvant combination plus IL-10 showed a marked decrease in antibody reactivity.

In addition, the non-dendritic peptide carrier constructs may be used in general to prolong the presence in circulation of any medicament such as peptide drugs.

Example 24

Non-dendritic peptide carriers derivatized with immunogenic peptides for specific induction of Th1-like and Th2-like responses

The non-dendritic peptide carriers derivatized with immunogenic peptides can be used in combination with cyto-kines for specific induction of Th1-like and Th2-like cellu-lar responses against any immunogenic agent. In connection with specific induction of such responses, the non-dendritic peptide carrier in combination with cytokines can thus be used for vaccination purposes to protect against pathogenic microorganisms, for therapy against infectious diseases, autoimmune diseases. Specific induction of Th1-like responses may be used for protection against diseases like leishmaniasis, tuberculosis, and possibly AIDS, and for therapy against allergic diseases etc., whereas specific induction of Th2-like responses may be used for protection against worm diseases and for therapy against toxic diseases (sepsis, meningitis, etc.).

Examples of the regulation of the immune system by non-dendritic peptide carriers are given below in which mice are immunized with a synthetic Human Immunodeficiency Virus-1 specific peptide or with *Leishmania major* specific peptides.

Materials and methods:

Mice were immunized with non-dendritic peptide carriers derivatized with one or more of the different model-peptides:

The HIV-1 peptide gp120,

aa152-176, (GEIKNCSFNISTSIRGKVQKEYAFF),

with known B- and T-cell epitopes.

L1: YDQLVTRVVTHEMAHA

This Leishmania specific peptide is reported to contain a T-cell epitope.

L2: EAEEAARLQA (H-2^{d} restricted Th2 epitope in Balb/C mice).

Cytokine peptides:
The IFN-gamma bioactive sequences:
IFN-gamma (1-39)
HGTVIESLESNNYFNSSGIDVEEKSLFLDIWRNWQKDG
IFN-gamma (95-133)
AKFEVNNPQVQRQAFNELIRVVHQLLPESSLRKRKRSRC

The TNF bioactive sequence (TNF70-80):
Pro-Ser-Thr-His-Val-Leu-Ile-Thr-His-Thr-Ile

Tuftsin peptide:
TKPR

Recombinant murine IFN-gamma, IL-4, IL-12, and TNF-alpha were purchased.

Synthetic peptide sequences of or recombinant gamma-interferon, IL-12 or IL-18 may be used for induction of Th1 responses. Synthetic peptide sequences of or recombinant IL-4, IL-5, or IL-13 may be used for induction of Th2 responses. Addition of adhesion molecules like B7-1, B7-2, P-selectin, or E-selectin may also affect the induction of Th1 and Th2 responses. The peptides and/or the cytokines and/or adhesion molecules may be used alone or in vehicles, with adjuvants, inserted into immunostimulating complexes, liposomes etc.

The dose of peptide as well as addition of different antigen presenting cells may also affect the induction of Th1 and Th2 responses.

Experimental procedure

Female 6-8 weeks old BALB/cJ mice (reported to be a Th2
responder mouse) were used in these studies. Mice were immu-
nized with the non-dendritic peptide carriers derivatized
with Leishmania or HIV-1 peptides without any conjugations.
Some mice were immunized intraperitoneally 3 times, day 0,
21, and 49, with 16 μg of peptide constructs, while some
other mice were immunized subcutaneously 3 times, day 0, 21,
and 49, with 16 μg of peptide constructs alone or mixed 1+1
with alum, with alum and recombinant cytokines or with
Freund's complete (1st immunization) or incomplete (2nd and
3rd immunization) adjuvant.

The mice were bled on days -1, 12, 33, and 61. Sera were
collected from the bleedings and tested in ELISA for IgG_{2a}
(marker of Th1) and IgG1 (marker of Th2) antibody reactivity
against the leishmania peptide or the HIV-1 gp-120 peptide.

The ELISA was performed as described in Example 20 in the
patent application.

Peripheral mononuclear cells, lymph node cells, and spleen
cells will be collected and stimulated *in vitro* with the
peptide, recombinant gp120 or PPD in optimal concentrations.
After 5-7 days of incubation, the supernatants will be har-
vested and tested for their content of gamma-interferon (as
an indicator of Th1 responses) and for their content of
interleukin-4 (as an indicator of Th2 responses), using
commercial ELISA-kits according to the instructions of the
manufacturer. Quantification of gamma-interferon mRNA and
interleukin-4 mRNA within the cells may also be undertaken.

Results

Immunization experiments with the leishmania peptide L1: Mice
were immunized with the non-dendritic peptide carrier deriva-

tized with L1 peptide plus alum alone or with Freund's complete adjuvant or with alum and one of the following recombinant murine cytokines: interferon-gamma, tumour necrosis factor (TNF), IL-12 or IL-4.

Fig. 25 shows the IgG1 response (putative Th2 response) of mice immunized with these L1 combinations. All mice produced IgG1 after 3 immunizations. Maximum responses were detectable after immunizations with recombinant TNF, recombinant IFN-gamma or Freund's complete adjuvant.

Mice immunized with recombinant TNF or recombinant IL-12 responded after 2 immunizations.

Fig. 26 shows the IgG2a response (putative Th1 response) of mice immunized with the same L1 combinations.

Mice immunized with recombinant TNF or Freund's complete adjuvant responded after 3 immunizations. Weak responses were detectable with the other L1 combinations.

A biphasic IgG2a response were recorded. Mice immunized with most of the combinations showed an IgG2a response after one immunization.

Conclusion:

Mice immunized with non-dendritic peptide carriers derivatized with L1 peptide and recombinant cytokines showed a strong antibody response to L1 peptide. All mice showed a strong Th2 like IgG1 response after 3 immunizations although many of them were immunized with a Th1 cytokine. The implications are that IgG1 and IgG2a may not be reliable markers of Th2 and Th1 responses, or that Th1 cytokines may also induce a Th2 response after repeated exposure. Th1 responses have been reported to develop before Th2 responses during experimental infections. We recorded a transient IgG2a response (putative Th1 response) after a single immunization. Some of these mice

did not show an IgG2a response after repeated immunizations. The strong IgG2a response detectable in two of the groups after 3 immunizations may be very different from the IgG2a response recorded after 1 immunization.

It was a novel finding that a synthetic peptide combination could be immunogenic after a single immunization. Such findings have only been recorded for DNA vaccines and attenuated microorganisms. An IgG2a response, after a single immunization, may be a marker of a Th1 response.

Mice were also immunized sc. and ip. with non-dendritic peptide carrier derivatized with L1 peptide and tuftsin. Mice showed a strong IgG1 response to L1-tuftsin construct sc. and a moderate response to L1-tuftsin construct ip. and to the L1 constuct alone sc. (fig. 27). The L1-tuftsin combinations did not induce an IgG2a response (fig. 28). Tuftsin is therefore interesting in that it only induces one branch of the immune system.

Mice were also immunized with non-dendritic peptide carriers derivatized with peptides covering 2 different sequences of IFN-gamma reported to mediate binding to the IFN-gamma receptor. Different combinations were immunogenic after 3 immunizations sc. (Fig. 29). Immunization with L1 and both IFN-gamma peptides induced the strongest IgG1 response, and this combination was immunogenic after 2 immunizations. This combination was also capable of inducing an IgG2a response after 3 immunizations (Fig. 30).

The different IFN-gamma peptides also induced a relatively weak IgG1 response after ip. immunizations (Fig. 31), but they did not induce an IgG2a response (Fig. 32).

Finally, mice were also immunized with non-dendritic peptide carriers derivatized with L1 peptide together with a peptide covering a sequence of TNF. The L1-TNF-peptide combination sc. induced a strong IgG1 response (Fig. 33), while ip.

immunizations did not induce an IgG1 response (data not
shown).

The sc. immunization with non-dendritic peptide carrier
derivatized with the L1-TNF peptide combination also induced
a biphasic IgG2a response comparable to the non-dendritic
peptide carrier derivatized with L1 peptide mixed with recom-
binant TNF, while ip. immunizations did not induce an IgG2a
response (Fig. 34).

The reproducibility of the results were tested with another
Leishmania peptide, L2, reported to be a Th2 inducing
peptide. The non-dendritic peptide carrier derivatized with
L2 peptide alone or combined with tuftsin or with the TNF
peptide all induced an IgG1 response after sc. immunizations,
while ip. immunizations were not immunogenic (Fig. 35). The
sc. immunizations did not induce an IgG2a response, but ip.
immunizations with the non-dendritic peptide carriers deriva-
tized with L2-tuftsin or L2-TNF-peptide combinations ip.
induced an IgG2a response after one immunization, but not
after 3 immunizations (Fig. 36).

Non-dendritic peptide carriers derivatized with the two IFN-
gamma peptides in combination, tuftsin or the TNF peptide,
were also tested in combination with the HIV-1 gp120 peptide.
Immunizations sc. stimulated IgG1 production with the TNF
peptide containing construct being the most immunogenic (fig.
37). Immunizations ip. were not immunogenic.

Immunizations sc. with non-dendritic peptide carriers deriva-
tized with the IFN peptides or the TNF peptide induced a
biphasic IgG2a response with responses detectable after the
first and the third immunization (Fig. 38). Immunizations ip.
were immunogenic after one immunization, but not after 3
immunizations. Tuftsin containing constructs did not induce
an IgG2a response.

Conclusion:

Several synthetic peptide combinations induced strong anti-
body responses without use of adjuvants or carriers. The fact
that so many different antigenic peptide cytokine peptide
combinations are strong immunogens is an entirely new find-
ing.

Example 25

**Non-dendritic peptide carriers derivatized with immunogenic
peptides for specific induction of enhanced cellular
responses**

Background:

The non-dendritic peptide carriers may include immunogenic
peptides in combination with bioactive cytokine peptides for
specific induction of enhanced cellular responses against any
immunogenic agent.

Alternatively, the non-dendritic peptide carrier derivatized
with immunogenic peptides can be used in combination with
cytokine molecules for specific induction of enhanced cellu-
lar responses against any immunogenic agent. The non-
dendritic peptide carrier in combination with cytokines can
thus be used for vaccination purposes to protect against
pathogenic microorganisms, for therapy against infectious
diseases, toxic diseases, autoimmune diseases, etc.

One example of this use of the non-dendritic peptide carrier
is given below in which mice were immunized with the non-
dendritic peptide carrier derivatized with a synthetic Human
Immunodeficiency Virus-1 specific peptide and with a single
copy of an IL-1 beta peptide or a tuftsin peptide included in
the structure.

Materials and methods:

An IL-1 beta bioactive sequence:

VQGEESNDK/Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys

or the TNF bioactive sequence:

Pro-Ser-Thr-His-Val-Leu-Ile-Thr-His-Thr-Ile

or the tuftsin sequence:

TKPR,

were synthesized N- or C-terminally within the non-dendritic peptide carrier derivatized with the peptide from HIV-1 gp120:

aa152-176:   GEIKNCSFNISTSIRGKVQKEYAFF

with known B- and T-cell epitopes.

The non-dendritic peptide carrier construct may be used alone, after conjugation to PPD, after insertion into immuno-stimulating complexes or liposomes, after addition of recom-binant cytokines, adhesion molecules, adjuvants, vehicles, carriers etc.

The same experimental and testing procedure as described in Example 20 above was followed.

Female 6-8 weeks old (C57BIxBALB/c)F1 mice were used in these studies.

Mice were immunized with the non-dendritic peptide carriers derivatized with the HIV-1 peptide with and without the IL-1 or tuftsin sequences without any conjugations. Mice were immunized subcutaneously 4 times, day 0, 21, 49, and 70, with

16 µg of peptide constructs alone or mixed 1+1 with Freund's complete (1st immunization) or incomplete (2nd and 3rd immunization) adjuvant.

The mice were bled on days -1, 12, 33, 61, and 82. Sera were collected from the bleedings and tested in ELISA for antibody reactivity against the linear HIV-1 gp-120 peptide.

The ELISA was performed as described in Example 20 in the patent application.

Results:

Experiments indicate that the non-dendritic peptide carrier derivatized with the HIV-1 gp120 peptide alone or with the inclusion of an IL-1 beta specific peptide sequence or the tuftsin sequence is immunogenic without the use of adjuvant. The non-dendritic peptide carrier structure may be acceptable for use in humans and provide a strong immunogenic stimulus to the immune system with applications in vaccine technology.

The non-dendritic peptide carrier derivatized with the gp-120 peptide (batch II) alone, in combination with Freund's adjuvant, in combination with the IL-1 peptide or in combination with the tuftsin peptide, induced antibody production after 3 immunizations sc. (Fig. 39). The strongest stimulator was the gp120 peptide plus tuftsin construct.

The non-dendritic peptide carrier derivatized with the gp120 peptide (batch IV), alone or in combination with the IL-1 peptide, induced antibody production after 3 immunizations sc. (Fig. 40).

Example 26

Non-dendritic peptide carriers for use as a therapeutic agent in therapy of cancer.

Non-dendritic peptide carriers may be used to cure or to inhibit cancers by direct neutralization of cancer cell growth and/or metastasis and by inducing neutralizing immune responses to the cancer cells.

To illustrate this application of the invention, examples are given in the following:

Materials and methods.

The following synthetic peptides will be synthesized:

Wildtype p53 peptide: KYMCNSSCM,

Mutant p53 peptide: KYICNSSCM,

and peptides from proteins such as MAGE, BAGE, GAGE, MART, melan, and tyrosinase or a 43 kDa protein, including

QDLTMKYQIF from the kDa protein, including
AAGIGILTV and ILTVILGVL from the MART-1/Melan-A protein
SAYGEPRKL from MAGE-1
SEIWRDIDF from tyrosinase
YRPRPRRY from GAGE-1
and the melanoma peptide YLEPGPVTA

The peptides will be presented as control peptides or in derivatized non-dendritic peptide carrier constructs with or without cytokines (TNF-alpha, GM-CSF), cytokine peptides or adhesion molecules such as B7 or B7 ligands CD28 or CTLA-4.

Female BALB/c mice (age 10-16 weeks) will be used. Mice will be immunized with 36 μg of peptide constructs, 1-3 times.

Tcyt activities:
3×10^6 immune spleen cells will be restimulated *in vitro* for 5-7 days with the peptides (app. 5 μM).

Cytolytic activity of the restimulated cells will be measured
by mixing the spleen cells with ^{51}Cr-labelled targets in
duplicate and incubated at 37°C for 4 hours. Supernatants
will be harvested with a harvesting system and radioactivity
will be counted in a gamma counter.

Percent-specific lysis will be calculated using the equation:
100 x ((experimental release-spontaneous release)/(maximal
release-spontaneous release)).

Protection against tumours:
Mice (5 per group) will be immunized with non-dendritic
peptide carrier constructs and challenged i.v. or s.c. with
cancer cells (1-9 x 10^5 sarcoma cells or mastocytoma cells).
Tumour growth will be monitored every 7 hours for at least 21
days.

Integrin adhesion molecule sequences and polymers of the RGD
sequence will be inserted into the non-dendritic peptide
carrier structure and tested for their ability to block
melanoma cell metastasis in BALB/c mice.

Cancer-cell specific sequences or carbohydrate structures
such as Le^x will be inserted into the non-dendritic peptide
carrier structure and used for diagnostical identification of
micro metastases, using ELISA methodologies as described in
Example 19.

Example 27

**Non-dendritic peptide carriers against autoimmune diseases:
Protection, treatment, and diagnosis**

Downregulation of the immune system in connection with e.g.
autoimmune diseases may be advantageously performed by
administrating relevant peptides linked to the non-dendritic
peptide carrier.

One example of this use for treatment of rheumatoid arthritis
is the preparation of a therapeutic composition for oral or
systemic intake in which the peptide *Mycobacterium bovis*
HSP60 aa 180-188 related to arthritis, having the sequence:

TFGLQLELT

or the peptide of HLA and DnaJ HSP having the sequence:

Q(K/R)RAA,

is linked to the non-dendritic peptide carrier.

Inbred Lewis rats and Fisher rats will be used. All rats will
be of 6-8 weeks of age. Arthritis will be induced by injec-
tion of *Mycobacterium tuberculosis* in Freund's complete
adjuvant. Rats will be injected intracutaneously at the base
of the tail with 100 μl of 10 mg/ml of *M.tuberculosis*. The
rats will be observed for clinical arthritis and scored by
grading of each paw from 0 to 4 based on erythema, swelling,
and deformity of the joint. All four legs will be scored, so
the highest score achievable will be 16.

Rats will be immunized 1-3 times intraperitoneally or subcu-
taneously with 5.35 and 200 μg of peptide constructs (plus
cytokine sequences and other immunomodulatory sequences in
some experiments) app. 35 days before induction of arthritis,
and scores for clinical arthritis will be obtained. Lymph
node lymphocytes will be collected 9 days after immunization,
and the lymphocytes will be tested for *in vitro* prolifer-
ation, IFN-gamma, and IL-4 production, in response to *M.tu-
berculosis* and to the peptide constructs.

Immunological responses to the peptide constructs will be
measured by testing for murine subclass Ig reactivities to
the peptides.

Another example is the linking of various peptides relevant in connection with encephalomyelitis (sclerosis) to the non-dendritic peptide carrier. Examples of such peptides include:

Rat T-cell receptor, having the peptide sequence:

DMGHGLRLIHYSYDDVNSTEKG,

rat T-cell receptor, having the peptide sequence:

ASSDSGNTE, and

the myelin basic protein Ac1-11: AcASQKRPSQRSK, where "Ac" denotes an acetyl group,

and the myelin basic protein p87-99: VHFFKNIVTPRTP with and without amino acid substitutions, and

NTWTTCQSIAFPSK or SKTSASIGSLCADARMYGVL or LINVIHAFQYV from the myelin proteolipid protein and PGYPIRALVGD from the myelin oligodendrocyte glycoprotein.

Experimental procedure:

Female Lewis rats, 6-12 weeks old, or (PL/JxSJL/J)F_1 mice or BALB/c mice or CBA mice or Biozzi ABH mice will be used.

Rats will be immunized 1-3 times intraperitoneally or subcutaneously with 5.35 and 200 µg of peptide constructs (plus cytokine sequences and other immunomodulatory sequences in some experiments) app. 35 days before or together with Freund's incomplete adjuvant supplemented with heat-killed 1 mg *Mycobacterium tuberculosis* (1+1) sc. At 30 days, each rat will be challenged with myelin basic protein (50 µg in complete Freund's adjuvant or similar proteins). Rats will be monitored daily from day 9 for clinical signs (tail weakness and limb paralysis). Alternatively, the rats may be fed with peptide constructs 2 days before challenge.

Lymph node lymphocytes will be collected 9 days after immunization, and the lymphocytes will be tested for *in vitro* proliferation, IFN-gamma, and IL-4 production, in response to *M.tuberculosis* and to the peptide constructs.

Immunological responses to the peptide constructs will be measured by testing for murine subclass Ig reactivities to the peptide constructs.

Another example is the linking of various peptides relevant in connection with diabetes to the non-dendritic peptide carrier. Examples of such peptides include:

Pseudomonas HSP60 peptides:
- VLGGGCALLRCIPACDSTLPANED
- PALDSLTPANEA

Glutamic acid decarboxylase peptidE GAD65$_{253-265}$: IARFKMFPEV-KEK

Glutamic acid decarboxylase peptidE GAD65$_{524-543}$: SRLSKVAPVIK-ARMMEYGTT.

HLA peptides TPQGRP(V/A/D/S)AEY or peptides containing this sequence,

and the regulatory peptide:
cks17:LQNRRGLDLLFLKEGGL.

Studies will be performed in Non-Obese Diabetic mice (NOD mice), 3-22 weeks old, which develop diabetes. NOD mice will be immunized s.c., i.p., orally or by nasal administration, 1-3 times (at weeks 3, 7, and 10) with peptide constructs (5-500 µg) alone or in combination with tuftsin or cytokines (peptides and recombinant proteins).

Immunological responses to the peptides will be measured by testing for murine subclass Ig reactivities to the peptides

and by testing lymph node and spleen cell proliferation and
gamma-interferon and interleukin-4 production *in vitro* in
response to peptide stimulation. Development of diabetes
after app. 12 weeks in the vaccinated mice will be monitored.

For assessment of diabetes, mice will be sacrificed and
pancreata and salivary glands will be fixed in formalin,
embedded in paraffin, and stained with hematoxylin/eosin and
scored for islet pathology. Mice will be tested weekly for
glucosuria by a commercial kit. Blood glucose will be
measured by using a blood glucose meter.

All such non-dendritic peptide carriers may be given in
combination with any immunomodulator or part thereof having
immunomodulator activity. Relevant immunomodulators include
interleukin-4, interleukin-10, and TGF-beta.

Another example is the linking of various peptides relevant
in connection with hen's egg allergy to the non-dendritic
peptide carrier. Examples of such peptides include:

EFRADHPFLF or peptides including this sequence from ovalbumin
inhibition of histamine release from basophils:

Whole blood (50 μl) diluted 1:2 in N-2-hydroxyethylpipera-
zine-N'-2-ethanesulphonic acid (20 mmol/l)-Tyrode's buffer
will be mixed at 4°C for 24 hours with 40 μl of peptide
constructs at concentrations 0-28,000 μg/ml dissolved in the
same buffer. Blood pretreated with peptides will be chal-
lenged for 30 minutes at 37°C with 50 μl of various dilutions
of allergen. The reaction will be stopped by centrifugation
at 1,500g at 4°C for 5 minutes. Histamine will be measured by
an Enzyme Linked Immunosorbent Assay or a radioimmunoassay.
The assays will be performed in duplicate. Spontaneous hista-
mine release from the cells will be subtracted from the
calculated histamine release. Percent inhibition of histamine
release by peptide constructs will be measured.

Example 28

The use of derivatized non-dendritic peptide carriers (DNDPC) for increasing blood-brain barrier permeability to peptides, drugs, diagnostic markers and other substances

Non-dendritic carrier peptides may be used to facilitate blood-brain barrier transfer for the purpose of brain and neurological therapy and diagnostics by intravenous (iv.) administration; this is illustrated in the following two planned examples.

A blood-brain barrier decreasing substance, including N-acet-ylglucosamine-N-acetyl muramic acid-Ala-Glu-Dap-Ala, where Dap is diaminopimelinic acid, (Spellerberg 1995), will be coupled as branch-moieties to an NDPC of the standard design described in Table 1.

Blood-brain barrier permeabilization will be analyzed by iv injection of FITC-dextran of 4, 10, 20, and 50 kD at fixed time points after iv injection of the DNDPC in rabbits as described (Spellerberg 1995). After anaestization cerebrospinal fluid will be collected and analyzed for fluorescence.

It is expected that the DNDPC will transiently increase BBB-permeability for molecules below 20 kD with a maximum permeability around 4 hours after DNDPC-administration.

In another experiment, it will be shown that a DNDPC is efficient in enhancing peptide-specific transport through the BBB of the DNDPC itself. The DNDPC will be prepared with multiple copies of one type of attached moiety, selected from

- YGGFM
- YGGFL
- Lys_N, in which N = 3-8

and another type of attached moiety, which in this example
will be a biotin-labelled model peptide:

biotin-LKYGGENKPGG.

In the planned experiment, rabbits will be injected iv., and
the cerebrospinal fluid will subsequently be analyzed for
avidinbinding by ELISA.

In all cases, the presence of biotin-peptide is expected to
increase when biotin-peptide is administered as a part of a
DNDPC compared to when administered alone.

Example 29

The use of derivatized non-dendritic peptide carriers (DNDPC) to inhibit cell-matrix attachment

This example demonstrates a therapeutic use of a derivatized
non-dendritic peptide carrier to stop the spread of cancerous
cells by inhibiting their matrix attachment. By including
cell-matrix binding interfering peptides as attachment pepti-
des in a DNDPC, a very efficient inhibitory substance is
expected to be obtained. In a planned experiment, peptides
containing sequences selected from
RGDS
RLDS
YIGSR
and multimers thereof (N = 2-4)(Fujii 1995, Nomizu 1993) will
be attached to an NDPC and tested for *in vivo* inhibition of
tumor growth and metastasis as compared to the corresponding
free peptides, using the experimental protocols detailed in
Nomizu et al. (1993) or equivalent protocols.

In short, for judgement of metastasis, mice will be injected
with detached melanoma cells in the presence or absence of
peptide by the tail vein, and, around 2½ weeks later, mice
will be sacrified and the number of lung colonies will be

enumerated. For tumor growth, the same cells will be adminis-
tered subcutaneously, followed by daily i.p. injections of
peptides. On day 10, mice will be sacrificed and the subcu-
taneous tumors will be weighed.

Example 30

**The use of derivatized non-dendritic peptide carriers (DNDPC)
containing carbohydrate antigen mimotopes for the production
of carbohydrate-specific antibodies for diagnosis and vacci-
nation; application to the Tn-antigen**

Peptides that mimick carbohydrate epitopes (peptide mimoto-
pes) can be defined by peptide library scanning methods with
suitable monoclonal antibodies against the carbohydrate
epitope in question. Such a mimotope peptide is expected to
be of use as an immunogen in the active immunotherapy of
cancer and HIV, and it is also expected to be a better
immunogen, especially with respect to induction of immunolog-
ical memory than the corresponding carbohydrate antigen.

The Tn-antigen (N-acetyl-D-galactosamine on serine or
threonine), which is associated with cancer and with HIV-
infection, and a mimotope corresponding to the Tn-carbohy-
drate antigen are planned to be employed in the following
illustrative way:

The Tn-mimotope peptide will be synthesized as an attached
antigenic peptide on a non-dendritic peptide carrier (NDPC)
as detailed in Example 1, using a RINK-MBHA solid phase. The
liberated whole complex will be used for immunizations of
mice and rabbits following normal immunization schemes (see
Example 20) and using Freund's incomplete adjuvant. In addi-
tion, derivatized NDPC in which is included or which is
administered together with a stimulating sequence, including
a tuftsin tetramer, fragments of cytokines or T-cell stimula-
tory peptides, may be used for immunizations without the
presence of adjuvants as described in Example 20. The result-

ing antibody response is analyzed for specificity against the
immunizing peptide *and* the Tn-epitope itself. The Tn-epitope
will be obtained from ovine submaxillary mucin which is
obtained from Sigma and then desialylated.

Good antibody reactivity is expected to be obtained against
the Tn-antigen, and it is also expected that these Tn-speci-
fic, peptide-directed antibodies will be able to block HIV-
infection and syncytium formation *in vitro*.

Example 31

**The use of antibodies against derivatized non-dendritic
peptide carriers (DNDPC) in an immunohistochemical diagnostic
assay for the prion disease bovine spongiform encephalitis**

The prion diseases are characterized by the presence of
misfolded prion proteins, presumably caused to misfold by the
influence of exogenous misfolded prion proteins. It has been
shown in sheep that abnormal prion proteins may be detected
pre-clinically by immunohistochemistry on tonsil biopsies
from scrapie-infected sheep (Schreuder 1996), using anti-
bodies raised against synthetic peptides corresponding to
abnormal prion protein sequences.

To develop the same type of diagnostic antibodies for the
bovine prion disease, the prion peptides will be synthesized
on a non-dendritic peptide carrier of the standard design
described in Example 1 and subsequently used for the produc-
tion of diagnostic antibodies in rabbits and mice by coinjec-
tion with adjuvant in a normal immunization scheme (see
Example 20). In addition, derivatized NDPC in which is
included or which is administered together with a stimulating
sequence, such as a tuftsin tetramer, fragments of cytokines
or T-cell stimulatory peptides, may be used for immunizations
without the presence of adjuvants as described in Example 20.
Each of the following prion peptides will be attached to a
non-dendritic peptide carrier, preferably by direct chemical

synthesis, in order to construct 5 different immunization DNDPCs:

GQGGTHGQWNKP
GQWNKPSKPKTN
GGLGGYMLGSAMSRPLIH
GSDYEDRYYRENMHRYPNQVYRPVDQYSNQNN
RESQAYYQRGAS

Antibodies produced against these DNDPCs will be tested on tonsil biopsies from normal cattle and from cattle in a preclinical state of spongiform encephalitis.

Shortly, tonsil biopsies will be pretreated with formic acid and hydrated autoclaving and will then be incubated with the antisera diluted in PBS followed by detection antibodies and staining (as detailed by van Keulen 1995). This treatment selectively enhances the immunoreactivity of the BSE-specific form of the prion protein.

Affected animals are expected to show antibody-positive biopsies before showing any clinical signs of the disease.

Example 32

Immunization against tuberculosis

The following synthetic peptides from the ESAT-6 antigen (Brandt, 1996) will be synthesized:

YQGVGGKWDATATELNNALQ

and

MTEQQWNFAGIEAAASAIQG

as control peptides and derivatized to the non-dendritic peptide carrier.

The peptides will be used for immunization of mice against
Mycobacterium tuberculosis.

Experimental procedure:

Mice:
C57BL/6J female mice, 8-12 weeks old, will be used.

Bacteria:
M.tuberculosis H37Rv will be grown at 37°C on Lowenstein-
Jensen medium or in suspension in Sauton medium enriched with
0.5% sodium pyrovate and 0,5% glucose.

Vaccination challenge and necropsy:
The mice will be immunized 3 times s.c. or i.p. with optimal
concentrations of synthetic peptide constructs with and
without 2 μg recombinant IL-12. After 12-14 weeks, the mice
will be challenged by an i.v. injection of 5 x 10^4 colony
forming units of *M.tuberculosis* suspended in PBS. The course
of disease will be compared with that for a corresponding
group of unimmunized mice during a period of 28 days. Enumer-
ations of bacteria in the spleen of infected mice will be
done by plating double serial 10-fold dilutions of spleen
homogenated on Lowenstein-Jensen medium. Colonies will be
counted after 3-4 weeks of incubation.

Lymphocyte cultures:
Spleen cells or lymph node cells will be isolated 2-3 weeks
after the previous immunization. And activated *in vitro* with
peptides or *M.tuberculosis* antigens. Cellular proliferation
will be investigated by pulsing cultures with tritiated
thymidine after 48 hours of incubation and then further
incubating for 22 hours, before the plates will be harvested
and processed for liquid scintillation counting.

Culture supernatants will be harvested from parallel cultures
after 24 hours, of incubation for determination of IL-2, IL-

4, and IL-5, and, after 48 hours, for determination of
interferon-gamma.

Cytokine assays:
The amounts of cytokines present in culture supernatants will
be quantified by commercially available ELISA kits.

Antibody titers of different IgG isotypes:
Serum samples will be collected after each immunization and
analyzed in two-fold dilutions on ELISA plates coated with
peptides or *M.tuberculosis* antigens. Reactivities will be
tested with peroxidase labelled goat anti-mouse IgG or anti-
mouse IgG1 or anti-mouse IgG2a according to the ELISA pro-
cedures described in Example 20.

Detection of cytokine mRNA:
Lymph node cells (10^6) will be lysed and total RNA purified.
RNA will be reversely transcribed into cDNA and subjected to
PCR amplification with specific primers for individual cytok-
ines. The PCR products will be hybridized with specific
labelled oligonucleotide probes for the cytokines in southern
blots.

Example 33

**The use of a derivatized non-dendritic peptide carrier for
the production of antibodies against outer membrane proteins
in *Actinobacillus pleuropneumoniae***

Mice were immunized with a derivatized non-dendritic peptide
carrier corresponding to type no. 2 and 3 in Table 1, carry-
ing as the branch peptide

AELGGQFHHKSENG (Tbp peptide 4)

from transferrin-binding protein type 2 from *Actinobacillus
pleuropneumoniae*, and another group of mice were immunized

with a derivatized non-dendritic peptide carrier correspond-
ing to type 3 in Table 1 carrying as the branch peptide

TEADYAKNRAVLEY (PalA peptide 5)

from proteoglycan-associated protein from the same bacterium.

The peptides were used either alone or in combination with
Freund's incomplete adjuvant. Subcutaneous injections were
used.

The mice were immunized 3 times at intervals of 14 days.
Bleedings were performed before the first immunization (0)
and 10 days after each immunization (1, 2, 3).

For comparison, another two groups of mice were immunized
with the corresponding peptides conjugated by SPDP through
cysteine to PPD ("Purified Protein Derivative") subsequent to
a BCG-priming as an example of a classical peptide-protein
conjugate immunization method.

The bleedings were analysed by ELISA using the various immun-
ization peptides as coating antigen. Peptides were coated
overnight at 4⁰C at 1 ug/ml in PBS on Maxisorp (Nunc) micro-
titre plates. Subsequent operations were performed at room
temperature. After blocking for 1 hour in PBS+1% BSA and wash
in PBS + Tween 20 (0.1%), mouse sera were applied 1/200 in
the blocking buffer and incubated for 1 hour. Subsequently,
plates were washed as above and developed by horse radish
peroxidase-conjugated rabbit anti-mouse immunoglobulins
(DAKO) for 1 hour followed by colour development by OPD and
peroxide (see Fig. 41 and 42).

The antibodies raised against the PPD-coupled Tbp-peptide 4
were cloned, and the resulting monoclonal antibody was tested
for its ability to recognise the Tbp-peptide 4 in different
presentations (see Fig. 43) by an indirect ELISA using the
different peptides as coating antigens.

Furthermore, reactivity with the proteins was analysed by Western blotting of whole cell extracts of *Actinobacillus pleuropneumoniae* (not shown).

In conclusion, the response against the derivatized non-dendritic peptide carrier with Freund's incomplete adjuvant is similar to the response obtained with PPD-conjugated peptides (see Fig. 41 and 42), and protein-reactive anti-bodies are obtained by both methods as shown by blotting (not shown). Furthermore, the monoclonal anti-PPD-Tbp-peptide 4 antibody selectively recognizes backbone-conjugated peptides, not free peptides whether in Iscoms or not (Fig. 43). This provides evidence that conjugation to the non-dendritic carrier peptide of this invention stabilizes the conformation of the branch-peptide at least as much as is obtained by classical conjugation methods.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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    (ii) TITLE OF INVENTION: Non-Dendritic Backbone Peptide Carrier

    (iii) NUMBER OF SEQUENCES: 52

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 26 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Pro Tyr Lys Cys Pro Glu Cys Gly Lys Ser Phe Ser Gln Lys Ser Asp
1   5   10   15

Leu Val Lys His Gln Arg Thr His Thr Gly
         20   25

(2) INFORMATION FOR SEQ ID NO: 2:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 4 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

        Thr Lys Pro Arg
        1

(2) INFORMATION FOR SEQ ID NO: 3:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 8 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

        Thr Lys Pro Arg Thr Lys Pro Arg
        1   5

(2) INFORMATION FOR SEQ ID NO: 4:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 12 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

        Thr Lys Pro Arg Thr Lys Pro Arg Thr Lys Pro Arg
        1   5   10

(2) INFORMATION FOR SEQ ID NO: 5:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 16 amino acids

```
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide




    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

    Thr Lys Pro Arg Thr Lys Pro Arg Thr Lys Pro Arg Thr Lys Pro Arg
    1               5               10              15


(2) INFORMATION FOR SEQ ID NO: 6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide




    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

    Thr Lys Pro Arg Thr Lys Pro Arg Thr Lys Pro Arg Thr Lys Pro Arg
    1               5               10              15

    Thr Lys Pro Arg
            20

(2) INFORMATION FOR SEQ ID NO: 7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide




    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

    Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu
    1               5               10

(2) INFORMATION FOR SEQ ID NO: 8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15 amino acids
```

                    (B) TYPE: amino acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear

              (ii) MOLECULE TYPE: peptide

              (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

        Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
        1   5   10   15

    (2) INFORMATION FOR SEQ ID NO: 9:

              (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 21 amino acids
                    (B) TYPE: amino acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear

              (ii) MOLECULE TYPE: peptide

              (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

        Phe Asn Asn Phe Thr Val Ser Phe Trp Leu His Arg Val Lys Val Ser
        1   5   10   15

        Ala Ser His Leu Glu
                    20

    (2) INFORMATION FOR SEQ ID NO: 10:

              (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 20 amino acids
                    (B) TYPE: amino acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear

              (ii) MOLECULE TYPE: peptide

              (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

        Asp Gln Val His Phe Gln Pro Leu Pro Pro Ala Val Val Lys Leu Ser
        1   5   10   15

        Asp Ala Leu Ile
                    20

    (2) INFORMATION FOR SEQ ID NO: 11:

```
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 21 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide




    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

    Asp Ile Glu Lys Lys Ile Ala Lys Met Glu Lys Ala Ser Ser Val Phe
    1                   5                   10                  15

    Asn Val Val Asn Ser
                20

(2) INFORMATION FOR SEQ ID NO: 12:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 18 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide




    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

    Lys Leu Leu Ser Leu Ile Lys Gly Val Ile Val His Arg Leu Glu Gly
    1                   5                   10                  15

    Val Glu


(2) INFORMATION FOR SEQ ID NO: 13:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 20 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide




    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

    Leu Asp Asn Ile Lys Gly Asn Val Gly Lys Met Glu Asp Tyr Ile Lys
    1                   5                   10                  15
```

```
Lys Asn Asn Lys
          20
```

(2) INFORMATION FOR SEQ ID NO: 14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 7 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

```
Val Ala Lys Leu Glu Ala Lys
1                   5
```

(2) INFORMATION FOR SEQ ID NO: 15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

```
Ala Val His Lys Leu Glu His Lys Val Ala Lys Leu Glu Ala Lys Gly
1               5                   10                  15

Lys Gly Lys Tyr
          20
```

(2) INFORMATION FOR SEQ ID NO: 16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

```
Leu Gln Gly Gln Asp Met Glu Gln Gln Val
```

1   5   10

(2) INFORMATION FOR SEQ ID NO: 17:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

    Asp Pro Lys Asn Tyr Pro Lys Lys Lys Met Glu Lys Arg Phe
    1   5   10

(2) INFORMATION FOR SEQ ID NO: 18:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 9 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

    Val Gln Gly Glu Glu Ser Asn Asp Lys
    1   5

(2) INFORMATION FOR SEQ ID NO: 19:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

    Gly Gly Thr Lys Gly Gly Gln Asp Ile Thr
    1   5   10

(2) INFORMATION FOR SEQ ID NO: 20:

```
    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 8 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide




   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

   Tyr Gly Leu Ala Glu Leu Lys Gly
   1               '   5

(2) INFORMATION FOR SEQ ID NO: 21:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 8 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide




   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

   Gly His Pro Leu Gln Lys Thr Tyr
   1               5

(2) INFORMATION FOR SEQ ID NO: 22:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 9 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide




   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

   Leu Thr Pro Leu Glu Glu Leu Tyr Pro
   1               5

(2) INFORMATION FOR SEQ ID NO: 23:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 11 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS: single
```

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

    Lys Asn Gly Met Leu Lys Gly Asp Lys Val Ser
    1   5   10

(2) INFORMATION FOR SEQ ID NO: 24:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 8 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

    Cys Lys Asn Lys Glu Lys Lys Cys
    1   5

(2) INFORMATION FOR SEQ ID NO: 25:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 6 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

    Leu Glu Arg Leu Leu Leu
    1   5

(2) INFORMATION FOR SEQ ID NO: 26:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Gly Glu Ile Lys Asn Cys Ser Phe Asn Ile Ser Thr Ser Ile Arg Gly
1   5   10   15

Lys Val Gln Lys Glu Tyr Ala Phe Phe
        20   25

(2) INFORMATION FOR SEQ ID NO: 27:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Trp Gly Cys Ser Lys Leu Ile Cys Thr Thr Ala Val Pro Trp Asn
1   5   10   15

(2) INFORMATION FOR SEQ ID NO: 28:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

Leu Gln Asp Gln Ala Arg Leu Asn Ser Trp Gly Cys Ala Phe Arg Gln
1   5   10   15

Val Cys His Thr
        20

(2) INFORMATION FOR SEQ ID NO: 29:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

```
Thr Leu Thr Lys Glu Tyr Glu Asp Ile Val Leu Lys Ser His Met Asn
1               5                   10              15

Arg Glu Ser Asp Asp
            20
```

(2) INFORMATION FOR SEQ ID NO: 30:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 19 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

```
Leu Lys Ser His Met Asn Arg Glu Ser Asp Asp Gly Glu Leu Tyr Asp
1               5                   10                  15

Glu Asn Ser
```

(2) INFORMATION FOR SEQ ID NO: 31:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 11 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

```
Pro Ser Thr His Val Leu Leu Thr His Thr Ile
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO: 32:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

```
Val Thr Glu Glu Ile
1               5
```

(2) INFORMATION FOR SEQ ID NO: 33:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

```
Val Thr Glu Glu Ile Val Thr Glu Glu Ile
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO: 34:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

```
Val Thr Glu Glu Ile Val Thr Glu Glu Ile Val Thr Glu Glu Ile
1               5                   10                  15
```

(2) INFORMATION FOR SEQ ID NO: 35:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

Val Tyr Lys Leu Glu Ala Lys Val Ala Lys Leu Glu Ala Lys
1   5   10

(2) INFORMATION FOR SEQ ID NO: 36:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 22 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

Cys Met Ser Asp Gly Ala Val Gln Pro Asp Gly Gly Gln Pro Ala Val
1   5   10   15

Arg Asn Glu Arg Ala Thr
            20

(2) INFORMATION FOR SEQ ID NO: 37:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

Met Ser Asp Gly Ala Val Gln Pro Asp Gly Gly Gln Pro Ala Val
1   5   10   15

(2) INFORMATION FOR SEQ ID NO: 38:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

```
Gln Pro Asp Gly Gly Gln Pro Ala Val Arg Asn Glu Arg Ala Thr
1               5               10              15
```

(2) INFORMATION FOR SEQ ID NO: 39:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 22 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

```
Asp Met Gly His Gly Leu Arg Leu Ile His Tyr Ser Tyr Asp Asp Val
1               5               10              15

Asn Ser Thr Glu Lys Gly
            20
```

(2) INFORMATION FOR SEQ ID NO: 40:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 9 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

```
Ala Ser Ser Asp Ser Gly Asn Thr Glu
1               5
```

(2) INFORMATION FOR SEQ ID NO: 41:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 8 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

```
Thr Leu Phe Gln Leu Glu Leu Thr
```

```
    1                5
```

(2) INFORMATION FOR SEQ ID NO: 42:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 18 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

```
Arg Ala Leu Thr Val Ala Glu Leu Arg Gly Ser Gly Asp Leu Gln Glu
1                5                   10                  15

Tyr Leu
```

(2) INFORMATION FOR SEQ ID NO: 43:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 15 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

```
Thr His Leu Val Ala Ile Gln Asn Lys Glu Glu Ile Glu Tyr Leu
1                5                   10                  15
```

(2) INFORMATION FOR SEQ ID NO: 44:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 9 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

```
Ala Ala Gly Ile Gly Ile Leu Thr Val
1                5
```

(2) INFORMATION FOR SEQ ID NO: 45:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 9 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

Tyr Leu Glu Pro Gly Pro Val Thr Ala
1   5

(2) INFORMATION FOR SEQ ID NO: 46:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

Leu Gln Asp Gln Ala Arg Leu Asn Ser Trp Gly Cys Ala Phe Arg Gln
1   5   10   15

Val Cys His Thr
            20

(2) INFORMATION FOR SEQ ID NO: 47:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 16 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

Trp Gly Cys Ser Gly Lys Leu Ile Cys Thr Thr Ala Val Pro Trp Asn
1   5   10   15

(2) INFORMATION FOR SEQ ID NO: 48:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

Leu Gly Ile Trp Gly Cys Ser Gly Lys Leu Ile Cys
1   5   10

(2) INFORMATION FOR SEQ ID NO: 49:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

Leu Gln Thr Met Val Lys Leu Phe Asn Arg Ile Lys
1   5   10

(2) INFORMATION FOR SEQ ID NO: 50:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

Asn Ser Val Asp Asp Ala Leu Ile Asn Ser Thr Lys Ile Tyr Ser Tyr
1   5   10   15

Phe Pro Ser Val
            20

(2) INFORMATION FOR SEQ ID NO: 51:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 16 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide




(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Lys
1               5                   10                  15


(2) INFORMATION FOR SEQ ID NO: 52:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 16 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide




(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

Pro Gly Ile Asn Gly Lys Ala Ile His Leu Val Asn Asn Glu Ser Ser
1               5                   10                  15
```

The following sequence nos. 53-130 are also included in the present invention:

53. VAKLEAKVAKLEAK
54. AVAKLEAKVAKLEAKGKGKY
55. KVAKLEAKVAKLEAKGKGKY
56. AVHKLEHKVAKLEAKGKGKYVQGEESNDK
57. KGKGKGL
58. KGKGKGLYL
59. WNSG
60. YLEN
61. YDQLVTRVVTHEMAHA
62. EAEEAARLQA
63. SGGKGSFDLEDV
64. AELGGQFHHKSENG
65. GMTAEDLQTRYN
66. TEADYAKNRAVLEY
67. CASQRDRFQVHNPHENDA
68. CKSQSGIEKTTRILHHANISESTQQN
69. CQATAKMAEEQLTTLHVRSEQQS
70. QGPGAPQGPGAPQGPGAPQGPGAP
71. GKGKGKGKGKGG
72. AIHKLEHKIAKLEAKGKGKY
73. PYKCPECGKSFSQKSDLVKHQRTHTG
74. KKK
75. KKKK
76. KKKKK
77. KKKKKK
78. KKKKKKK
79. KKKKKKKK
80. LKYGGENKPGG
81. YGGFM
82. YGGFL
83. RGDS
84. RLDS
85. YIGSR
86. GQGGTHGQWNKP
87. GQWNKPSKQKTN

88. GGLGGYMLGSAMSEPLIH

89. GSDYEDRYYRENMHRYPNQVYYRPVDQYSNQNN

90. RESQAYYQRGAS

91. KYMCNSSCM

92. KYICNSSCM

93. QDLTMKYQIF

94. ILTVILGVL

95. SAYGEPRKL

96. SEIWRDIDF

97. YRPRPRRY

98. TFGLQLELT

99. QKRAA

100. QRRAA

101. DMGHGLRLIHYSYDDVNSTEKG

102. ASQKRPSQRSK

103. VHFFKNIVTPRTP

104. NTWTTCQSIAFPSK

105. SKTSASIGSLCADARMYGVL

106. LINVIHAFQYV

107. PGYPIRALVGD

108. VLGGGCALLRCIPACDSTLPANED

109. PALDSLTPANEA

110. IARFKMFPEVKEK

111. SRLSKVAPVIKARMMEYGTT

112. TPQGRPVAEY

113. TPQGRVAAEY

114. TPQGRVDAEY

115. TPQGRVSAEY

116. EFRADHPFLF

117. HGTVIESLESLNNYFNSSGIDVEEKSLFLDIWRNWQKDG

118. AKFEVNNPQVARAAFNELIRVVHQLLPESSLRKRKRSRC

119. YQGVQQKWDATATELNNALQ

120. MTEQQWNFAGIEAAASAIQ

121. LQNRRGLDLLFLKEGGL

122. RGD

123. GACRGDCLGA

124. GRGDSP

125. CRGDCL

126. CRGDCA
127. CVLNGRME
128. NGRAHA
129. DGRAHA
130. ALNGREESP

CLAIMS

1. A non-dendritic peptide carrier coupled through a linker to a solid phase forming a non-dendritic peptide-solid phase complex, the non-dendritic peptide carrier comprising 10-50 amino acids capable of forming a secondary structure in a benign buffer after liberation from the solid phase, the non-dendritic peptide-solid phase complex further comprising an immunogenic substance and/or an immune mediator coupled on the non-dendritic peptide carrier.

2. A non-dendritic peptide carrier as defined in claim 1 further comprising a covalently bound lipidic moiety.

3. A non-dendritic peptide carrier according to claim 1 or 2 comprising 10-26 amino acids capable of forming a secondary structure in a benign buffer after liberation from the solid phase.

4. A non-dendritic peptide carrier according to any of the preceding claims, wherein the secondary structure is selected from the group α-helices, β-strand, β-turns, γ-turns, zinc-finger structures and combinations thereof.

5. A non-dendritic peptide carrier according to claim 4, wherein the secondary structure is an α-helix.

6. A non-dendritic peptide carrier according to any of the preceding claims, wherein the secondary structure is a result of the inclusion in the peptide of one or more α-helical-, β-strand-, turn- or zinc-finger-inducing sequences of amino acids, respectively, or combinations thereof.

7. A non-dendritic peptide carrier according to any of the preceding claims, wherein the non-dendritic peptide carrier is linked by its C-terminus to a dibranching molecule presenting two molecules of the peptide in parallel.

8. A non-dendritic peptide carrier according to any of the preceding claims wherein the peptide exists as a monomer in a benign aqueous solution.

9. A non-dendritic peptide carrier according to any of the preceding claims and which forms an amphipathic helix in a benign aqueous solution, giving rise to an intramolecular anti-parallel arrangement of two α-helices joined by a turn in a monomer supersecondary hairpin structure.

10. A non-dendritic peptide carrier according to any of claims 1-5 existing as a dimer in a benign aqueous solution.

11. A non-dendritic peptide carrier according to claim 10 and forming amphipathic α-helices in aqueous solutions, giving rise to parallel homodimers of the α-helix coiled-coil type.

12. A non-dendritic peptide carrier according to any of the preceding claims, which carries at least 2 attachment points being derivatizable and accessible functional groups.

13. A non-dendritic peptide carrier according to claim 12 in which the attachment points are chosen from the group of ϵ-amino groups or derivatized ϵ-amino groups in the side-chain of lysine or lysine analogous, such as ornithine, α,γ-diaminobutyric acid or α,β-diaminopropinyl acid and a free α-amino group.

14. A non-dendritic peptide carrier according to claim 13 further comprising a subclass of ϵ-amino groups in the side-chain of other lysine residues and a free α-amino group, said subclass comprising at least one such amino group being protected by a protecting group cleavable by a chemical treatment orthogonal to both Fmoc- and Boc-cleaving chemical treatments.

15. A non-dendritic peptide carrier according to claim 14 comprising as attachment points, a double-functional attachment point, and/or a multiple-functional attachment point.

16. A non-dendritic peptide carrier according to claim 15 in which one of the functional groups of the double-functional attachment points or at least one of the functional groups of the multiple-functional attachment points is reversibly blocked.

17. A non-dendritic peptide carrier according to any of the claims 11-15 in which the side-chain functional groups serving as attachment points are derivatized with secondary structure supporting and derivatizable building blocks.

18. A non-dendritic peptide carrier according to any of the claims 11-15 in which the side-chain functional groups serving as attachment points are derivatized with a dibranching residue being selected from the structures shown in Fig. 4A and Fig. 4B.

19. A non-dendritic peptide carrier according to any of the preceding claims which comprises at least two free carboxylic acids or amino groups located in a side-chain attached near to the C-terminal amino acid.

5

20. A non-dendritic peptide carrier according to any of the preceding claims, comprising as a lipidic moiety at least one alkyl- and/or alkenyl chain, preferably in the form of a fatty acid bound covalently to the N-terminus of the peptide or to an amino acid side-chain.

10

21. A non-dendritic peptide carrier according to claim 20 wherein the carbon chain of the alkyl- or alkenyl chain comprises about 4-25 carbon atoms such as 6-20 carbon atoms, preferably 7-17 carbon atoms in length.

15

22. A non-dendritic peptide carrier according to claim 21 wherein the carbon chain of the lipidic moiety comprises palmitic acid or myristic acid or mixtures thereof.

23. A non-dendritic peptide carrier according to any of the claims 20-22 in which at least one lipidic moiety is bound as a thioester to the peptide, preferably through a cysteine side chain thiol group.

24. A non-dendritic peptide carrier according to any of the claims 20-23 in which the lipidic moiety is the immunostimulatory $palm_3$-Cys-molecule shown in Fig. 4C.

25. A non-dendritic peptide carrier according to any of the claims 20-24 in which the lipidic moiety is coupled to the side chains of amino acids selected from lysine and serine being of alternating chirality, a D-amino acid being followed by a L-amino acid and a L-amino acid being followed by a D-amino acid.

26. A non-dendritic peptide carrier according to any of the preceding claims comprising 15-50 amino acids and comprising in its sequence repeated "heptads" having the form "abcdefg".

27. A non-dendritic peptide carrier according to claim 26 wherein the repeated "heptads" are located in its inner sequence, that is the sequence located within the segment of the peptide carrier corresponding to the segment from amino acid 2-5 to n-(2-5), preferably to the segment from amino acid 3 to n-3, where n is the number of amino acids in the peptide.

28. A non-dendritic peptide carrier according to any of claims 26-27 in which lysine residues occupying "e" and "g" positions are orthogonally side-chain protected compared to lysine residues occupying "b", "c" and "f" positions.

29. A non-dendritic peptide carrier according to any of claims 26-28, in which at least one "f" position is occupied by C.

5   30. A non-dendritic peptide carrier according to any of the preceding claims which comprises one or more peptide moieties selected from

          i) [TKPR]$_N$, in which N is preferably from 1-5 (tuftsin
10   oligomer), muramyldipeptide (N-acetyl-muramyl-L-alanyl-D-isoglutamine) or variants thereof;

          ii) a T-cell stimulatory peptide selected from QYIKANSKFIGITE (tetanus toxoid 830-843) and FNNFTVSFWLHRVKVSASHLE (tetanus
15   toxoid 947-967), DQVHFQPLPPAVVKLSDALI (Mycobacterium tuberculosis 38 kD antigen 350-369), DIEKKIAKMEKASSVFNVVNS (Plasmodium falciparum circumsporozoite protein 378-398), KLLSLIKGVIVHRLEGVE, measles virus F-protein 286-302, LDNIKGN-VGKMEDYIKKNNK (Plasmodium falciparum MSP-1, 260-279), LQTMVK-
20   LFNRIK, NSVDDALINSTKIYSYFPSV, QYIKANSKFIGITELK, and PGINGKAIHLVNNESS;

          iii) a T-cell stimulatory peptide selected from poly T-cell-epitope constructs wherein the T-cell-epitope elements are
25   arranged in a substantially linear construction comprising interposed minimal T-cell epitope peptide segments, preferably without flanking sequences;

          iv) a cytokine derived peptide selected from
30   IFN-gamma(1-39) HGTVIESLESNNYFNSSGIDVEEKSLFLDIWRNWQKDG, IFN-gamma(95-133) AKFEVNNPQVQRQAFNELIRVVHQLLPESSLRKRKRSRC, TNF (70-80) PSTHVLITHTI, and IL-1 beta (163-171) VQGEESNDK

35   v) combinations, including copies thereof.

31. A non-dendritic peptide carrier according to any of the preceding claims which also comprises at least one PNA moi-

ety, said PNA comprising a monomer sequence binding a speci-
fic DNA-molecule by hybridization.

32. A non-dendritic peptide carrier according to claim 1-30
comprising at least one functional DNA-intercalator moiety.

33. A non-dendritic peptide carrier according to claim 1-30
comprising at least one DNA oligonucleotide moiety.

34. A non-dendritic peptide carrier according to claim 33
comprising as the DNA moiety an oligonucleotide comprising
the sequence (5')PuPuCpGPyPy(3'), in which Pu is a purine
base and Py is a pyrimidine base.

35. A non-dendritic peptide carrier according to claim 1-30
comprising a polycationic moiety, preferably poly-lysine, the
number of lysines preferably from about 10 to about 15.

36. A non-dendritic peptide carrier according to any of the
preceding claims substituting the solid-phase to from about
0.001 to about 5, preferably such as from about 0.01 to about
1, more preferred from about 0.02 to about 0.08, still more
preferred from about 0.04 to about 0.06, most preferred from
about 0.05 to about 0.1 mmoles pr. gram of solid phase.

37. A non-dendritic peptide carrier according to any of the
preceding claims in which the chemical treatment liberating
the peptide from the solid-phase bound linker is orthogonal
to the chemical treatment used for the cleavage of protecting
groups from the intended attachment points in the side-chains
of the peptide.

38. A non-dendritic peptide carrier according to claim 37
wherein the peptide is linked to the solid phase through a
linker releasing the peptide on contact with water, preferab-
ly a glycolic acid linker of the structure given in Figure 4D
or a linker releasing the peptide through diketopiperazine
formation.

39. A non-dendritic peptide carrier according to any of the preceding claims comprising 10-26 amino acids and containing a lipidic moiety located at the C-terminus or near the C-terminus being preferably palmitic or myristic acid, said peptide containing no free and accessible side-chain functionalities but an unprotected N-terminus α-amino group.

40. A non-dendritic peptide carrier according to any of the claims 1 or 2 comprising 5-50 amino acids said peptide comprising a covalently bound lipidic moiety, preferably palmitic or myristic acid, located at the N-terminus or near the N-terminus, said peptide being attached to the solid phase by a hydrazine-cleavable linker to form a solid phase complex.

41. A non-dendritic peptide carrier according to claim 30, wherein the solid-phase is further derivatized in a defined ratio with a selectively cleavable linker, said linker being cleavable orthogonally to the non-dendritic peptide-linker and designed as a peptide anchoring moiety.

42. A non-dendritic peptide carrier according to any of the preceding claims further comprising a substance having characteristic and measurable spectral or radioactive properties such as UV-absorbing properties, visibly absorbing or fluorescent properties.

43. A non-dendritic peptide carrier according to any of the preceding claims which is non-cyclic.

44. A method for preparing a non-dendritic peptide carrier comprising an immunogenic substance or immune mediator coupled thereon comprising the steps of

i) synthesizing a non-dendritic peptide carrier as defined in any of the preceding claims by chemical solid-phase synthesis on a linker, and

ii) synthesizing the immunogenic and/or immune mediator substance directly on the non-dendritic peptide carrier, and

iii) cleaving the non-dendritic peptide carrier from the solid phase.

45. The method according to claim 44 wherein step ii) further comprises the covalent attachment of other moieties to the peptide carrier by derivatizable groups thereon.

46. The use of a non-dendritic peptide-solid phase complex as defined in any of claims 1-43 as a scaffold for the production of chemical derivatives, characterised by covalently attaching molecules at attachment points, the molecules being selected from polypeptides, carbohydrates, haptens, glycopeptides, lipopeptides, DNA, RNA, PNA, proteins and glycoproteins and combinations thereof.

47. The use of a non-dendritic peptide-solid phase complex as defined in any of claims 1-46 as a scaffold for the stepwise Fmoc- or Boc-based solid-phase peptide synthesis of peptide moieties with defined sequences on the attachment-points of the solid-phase bound non-dendritic peptide.

48. The use of a non-dendritic peptide carrier as defined in any of claims 1-43 as a scaffold-peptide for the incorporation into a Immunostimulating Complex (Iscom) resulting in a non-dendritic peptide carrier-Iscom complex.

49. The use of a non-dendritic peptide carrier-Iscom complex according to claim 48 for the chemical coupling of antigenic substances in an aqueous solution by methods of conjugating peptides to proteins known in the art.

50. A non-dendritic peptide carrier as described in any of claims 1-43 derivatized with one or more peptides having fibronectin-like, laminin-like or vitronectin-like binding activities.

51. A derivatized non-dendritic peptide carrier according to
claim 50 wherein the peptide having fibronectin-like, lami-
nin-like or vitronectin-like binding activities comprises in
its sequence RGD, GACRGDCLGA, GRGDSP, CRGDCL, CRGDCA, CVLNGR-
ME, NGRAHA, DGRAHA, ALNGREESP, YIGSR, RLDS, or variants
thereof.

52. The use of a derivatized non-dendritic peptide carrier
according to any of claim 50-51 for the promotion of cell-
attachment to plastic surfaces, comprising coating the plas-
tic surface with the derivatized non-dendritic peptide car-
rier before attaching the cells.

53. The use of a derivatized non-dendritic peptide carrier
according to claim 50-52 for the inhibition of tumor growth
and metastasis.

54. The use of a derivatized non-dendritic peptide carrier
according to any of claims 50-53 for the promotion of wound
healing by coating the peptide onto the damaged tissue.

55. The use of a derivatized non-dendritic peptide carrier as
defined in any of claims 1-43 for the selection of specific-
ally binding aptamers.

56. A diagnostic component for detecting a molecule or a
substance comprising a derivatized non-dendritic peptide
carrier as defined in any of claims 1-43 wherein a diagnostic
agent is linked.

57. A diagnostic component according to claim 56, wherein the
diagnostic agent is an antigen or an antibody.

58. A diagnostic component according to any of claims 56-57
for detecting a molecule which is an aptamer.

59. A diagnostic component according to any of claims 56-58, in which the diagnostic component comprises at least two different diagnostic agents capable of detecting the same or different molecules.

60. A diagnostic component according to any of claims 56-59, in which the diagnostic agent is a molecule selected from the group consisting of a polypeptide; lipopolypeptide; a glyco-polypeptide; a phospholipid: a carbohydrate: a lipopolysacch-aride; a nucleotide sequence such as a DNA sequence or a RNA sequence; PNA; or any combinations or modifications thereof.

61. A diagnostic component according to any of claims 56-60, in which the amount of the diagnostic agent is effective to detectably react with said molecule to be detected and to which the diagnostic agent is capable of binding.

62. The use of a diagnostic component according to any of claims 56-61 for detection of a molecule, in which the diag-nostic composition is incubated with said molecule for a time sufficient for the diagnostic composition to react with the subject and forming a complex and detecting the presence of bound molecule by subjecting said complex to detecting means.

63. The use according to claim 62, for detection of a mol-ecule derived from or indicative of pregnancy, of a disease, such as an infectious disease, an autoimmune disease, a cancerous disease or any other disease wherein an indicative molecule is known.

64. The use according to any of claims 62-63 for detection of a molecule in a sample derived from tissue or tissue extract, a cell culture or an mammal, including a human being.

65. The use according to claim 64, in which the sample is derived from tissue such as a biopsy, or an extract from tissue, a cell, or a cell culture from said mammal.

66. A method of diagnosing a disease or pregnancy in an mammal including a human being, comprising a diagnostic component according to any of claims 56-61 to the mammal, the diagnostic agent detecting in vivo a molecule derived from or indicative of the disease or pregnancy of said mammal.

67. A method of diagnosing a disease or pregnancy in an mammal including a human being, comprising incubating a diagnostic component according to any of claims 56-61 in a sample obtained from said mammal, the diagnostic agent detecting a molecule derived from or indicative of the disease or pregnancy of said mammal and present in the sample.

68. A method according to claim 66 or 67, for detection of a molecule derived from or indicative of an infectious disease, an autoimmune disease, a cancerous disease or the like.

69. A method according to any of claims 66-67, for detection of a molecule in a sample derived from tissue such as a biopsy, or an extract from tissue, a cell, or a cell culture from said mammal.

70. A method according to claim 69, in which the sample is derived from serum, plasma, whole blood, cerebrospinal fluid, seminal or vaginal fluids, saliva, exudates, urine, faeces, or the like.

71. A diagnostic kit comprising a diagnostic component according to any of claims 55-61.

72. A vaccine component comprising a non-dendritic peptide carrier as defined in any of claims 1-43 to which at least one immunogenic agent or mediator is attached.

73. A vaccine component according to claim 72, in which the immunogenic agent is a polypeptide, a glycopeptide, a lipopeptide, a phospholipid, a polysaccharide, a lipopolysaccar-

ide, a carbohydrate, a nucleotide sequence, PNA or any combi-
nation or modifications thereof.

74. A vaccine component according to any of claims 72-73, in
which the immunogenic agent or mediator is a mixture of
peptides.

75. A vaccine component according to any of claims 72-74
wherein the immunogenic agent or mediator is capable of
affecting the immunogenic effect or the reaction of the
immune system exposed to the vaccine component.

76. A vaccine component according to claim 76, in which the
immune mediator is a tuftsin, a cytokine, an adhesion mol-
ecule, or a part or modification thereof.

77. A vaccine component according to any of claims 72-74 in
which the vaccine component is attached to a second carrier,
such as an Immunostimulating Complex (Iscom), a liposome or
an immune microparticle, optionally in combination with a
mediator or a part thereof having mediator activity and
attached to the second carrier.

78. A vaccine composition comprising at least one vaccine
component according to any of claims 72-77.

79. A vaccine composition according to claim 78 comprising an
effective amount of the vaccine component to confer increased
resistance to one or more infection(s) in the mammal, the
composition optionally further comprising a pharmaceutically
acceptable carrier, vehicle, enhancer or adjuvant, or mix-
tures thereof.

80. A method of immunizing an mammal, including a human being
against a disease, the method comprising administering to the
mammal an immunogenically effective amount of the vaccine
composition according to claim 78 or 79.

81. A method of immunizing an mammal, including a human, according to claim 80 comprising administering the vaccine composition orally, nasally, rectally, subcutaneously, intradermally, or intramuscularly, or on any mucosal surface.

82. A therapeutic component comprising a non-dendritic peptide carrier as defined in any of claims 1-43 to which at least one therapeutic or prophylactic agent is attached.

83. A therapeutic component according to claim 82 in which the therapeutic or prophylactic agent is a polypeptide, a glycopeptide, a lipopeptide, a phospholipid, a polysaccharide, a lipopolysaccaride, a carbohydrate, a nucleotide sequence, PNA, or any combination or modifications thereof.

84. A therapeutic component according to any of claims 82-83 further comprising at least one mediator capable of controlling or enhancing the effect of the therapeutic or prophylactic agent linked to the carrier.

85. A therapeutic component according to claim 84, in which the mediator is a tuftsin, an immunomodulator including a cytokine, an adhesion molekule, or a part or modification thereof.

86. A therapeutic component according to any of claims 82-85 in which the therapeutic component is attached to a second carrier, such as an Immunostimulating Complex (Iscom), a liposome or a microparticle, optionally in combination with a mediator or a part thereof having mediator activity and attached to the second carrier.

87. A therapeutic component according to any of claims 82-86 further comprising a targeting molecule capable of binding to a target substance present at a specific location in the mammal thereby directing the therapeutic component to said specific location where the therapeutic component is to exert its effect.

88. A therapeutic component according to claim 87, in which the targeting molecule is an antibody.

89. A therapeutic component according to any of claims 87-88 capable of preventing, including preventing relapse, or treating a disease or capable of preventing or disrupting pregnancy.

90. A pharmaceutical composition comprising a therapeutic component according to any of claims 82-89 optionally together with a pharmaceutically acceptable carrier.

91. A pharmaceutical composition according to claim 90 for treatment or prevention of a disease, such as an infectious disease, a cancerous disease or an autoimmune disease.

92. Use of a therapeutic component according to any of claims 82-89 for the preparation of a pharmaceutical composition.

93. A method of treatment and/or prevention of a disease, comprising administering to a patient in need thereof a therapeutically or prophylactically effective amount of a pharmaceutical composition according to any of claims 90-91.

94. Use of a therapeutic component according to any of claims 82-89 for the preparation of a pharmaceutical composition.

95. A detecting component for detecting a molecule or a substance, the component comprising a non-dendritic peptide carrier as defined in any of claims 1-43 to which a detecting agent is linked.

96. A vaccine component according to any of claims 72-79 in which the immunogenic agent is the peptide aa 152-176 from HIV-1 gp120 having the amino acid sequence: GEIKNCSFNISTSIRG-KVQKEYAFF.

97. A vaccine component according to any of claims 72-79 in which the immunogenic agent or mediator is the peptide from HIV-1 gp41 comprising the following amino acid sequence: LERLLL.

98. A vaccine component according to any of claims 72-79 in which the immunogenic agent is YDQLVTRVVTHEMAHA.

99. A vaccine component according to any of claims 72-79 in which the immunogenic agent is EAEEAARLQA.

100. A vaccine component according to any of claims 72-79 in which the immune mediator is IFN-gamma(1-39) HGTVIESLESNNYFN-SSGIDVEEKSLFLDIWRNWQKDG.

101. A vaccine component according to any of claims 72-79 in which the immune mediator is IFN-gamma(95-133) AKFEVNNPQVQRQ-AFNELIRVVHQLLPESSLRKRKRSRC.

102. A vaccine component according to any of claims 72-79 in which the immune mediator is the TNF bioactive sequence (TNF70-80):
Pro-Ser-Thr-His-Val-Leu-Ile-Thr-His-Thr-Ile.

103. A vaccine component according to any of claims 72-79 in which the immune mediator is the Tuftsin peptide TKPR or oligomers thereof.

104. A vaccine component according to any of claims 72-79 in which the immune mediator is the IL-1 beta bioactive sequence: VQGEESNDK/Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys.

105. A vaccine component according to any of claims 72-79 in which the immunogenic agent is the EBA 175-peptide: TLTKEYED-IVLKSHMNRESDD.

106. A therapeutical component according to any of claims 82-94 in which the therapeutical agent is KNGMLKGDKVS.

107. A therapeutical component according to any of claims 82-94 in which the therapeutical agent is CKNKEKKC.
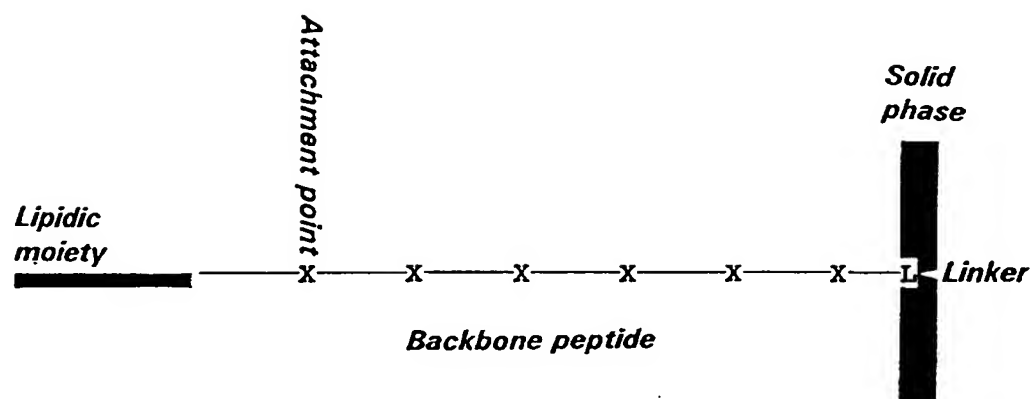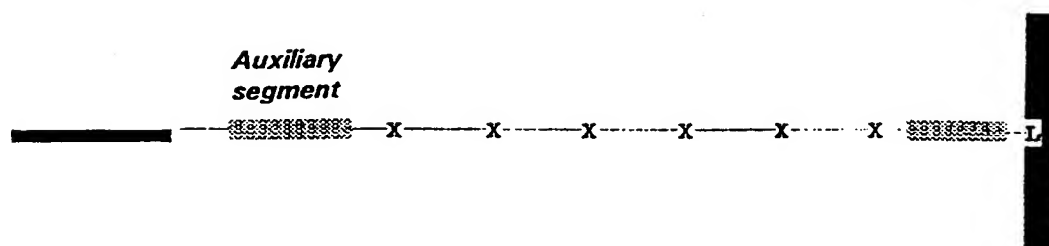
Fig. 1A

Fig. 1B

Branch
peptide

Fig. 1C

Auxiliary
segment

Fig. 1D

Auxiliary
segment

Fig. 1E

Fig. 2A

Fig. 2B

Fig. 3

H2NH2CH2CHNOCH2CO.   OCH2CONHCH2CH2NH2

CONHCH2CH2COOH

H2Dab-OH

Fig. 4A

RHN(CH2)3
            NCH2COOH   where R=
RHN(CH2)3

Fmoc

Fig. 4B

$$CH_2-CH-CH_2-S-CH_2-CH-CO\cdot$$
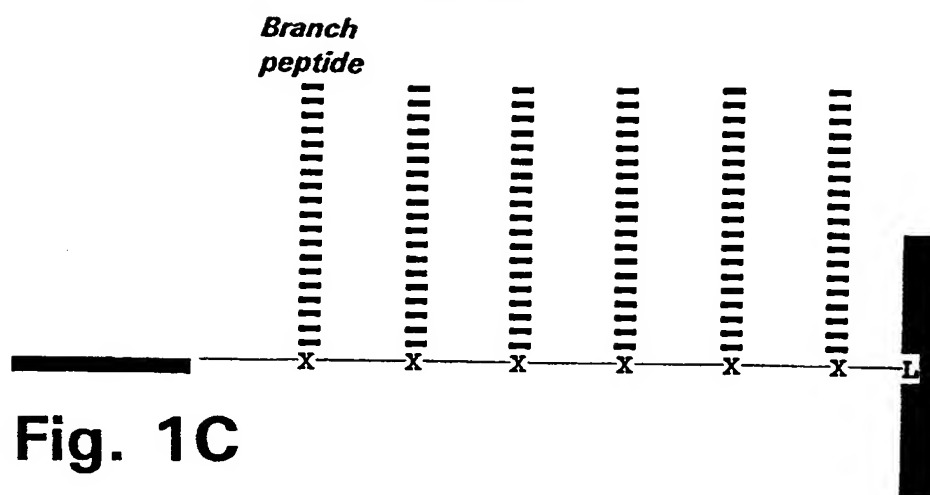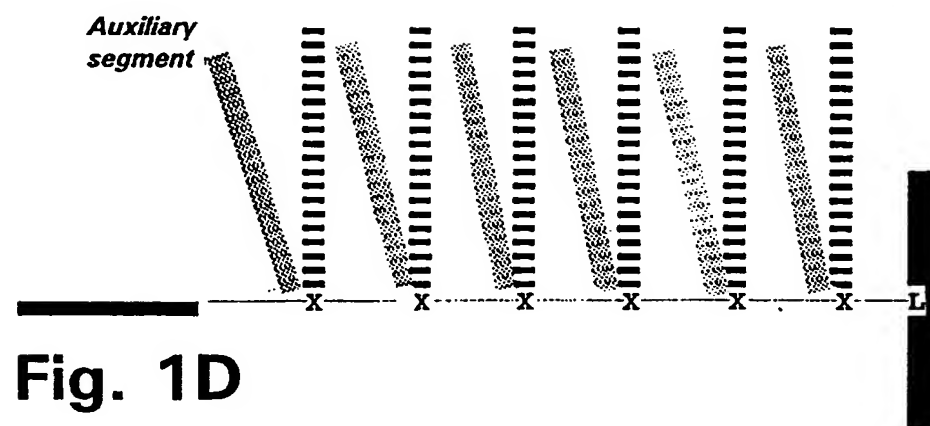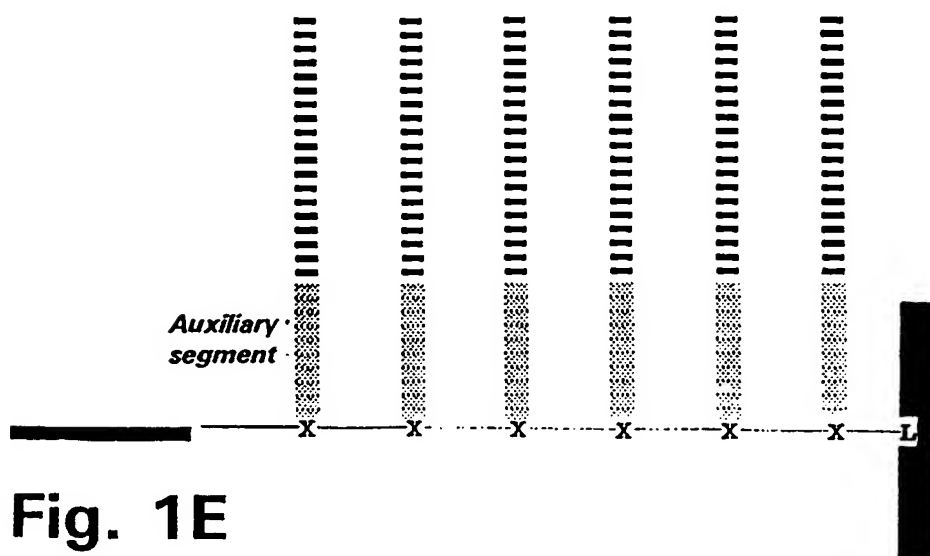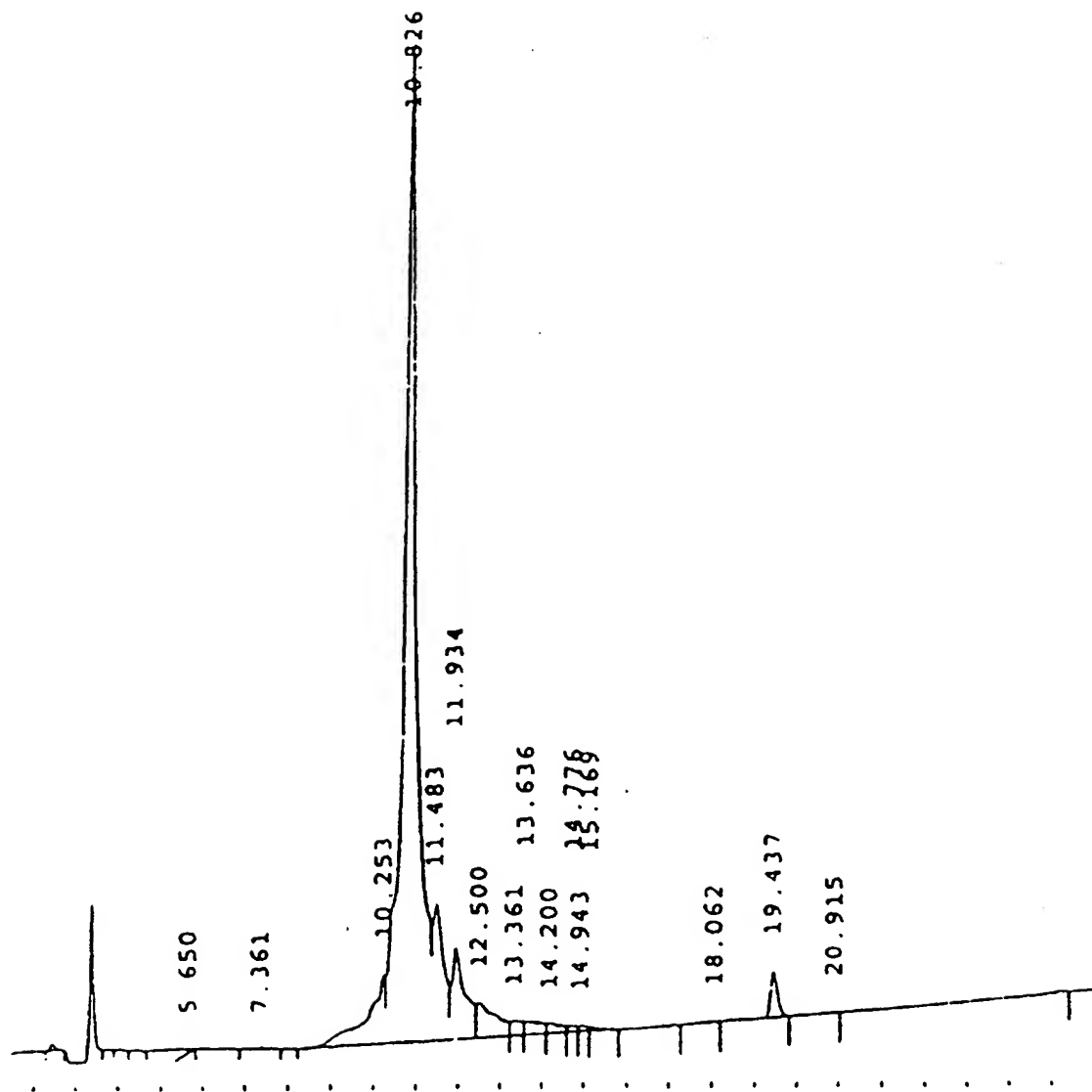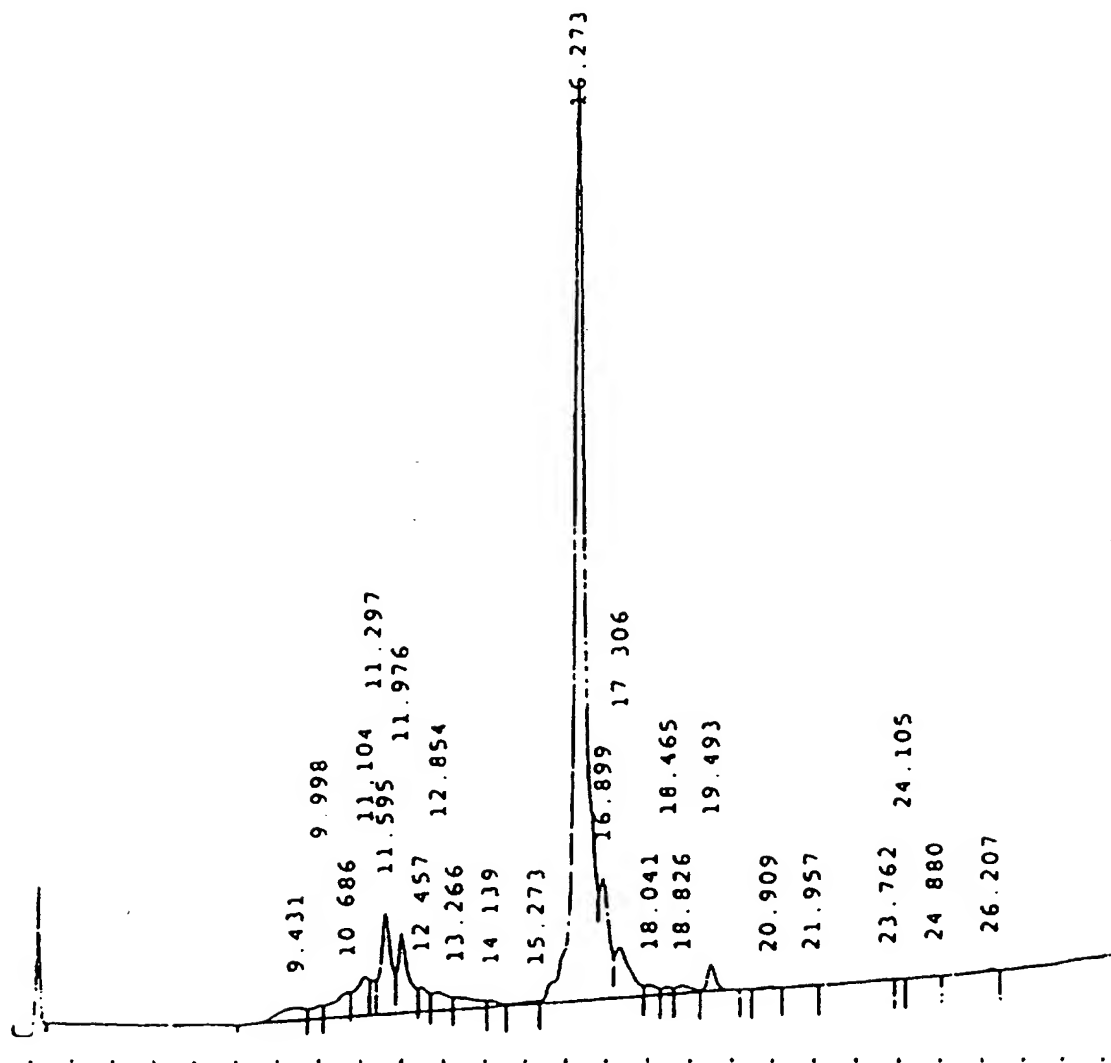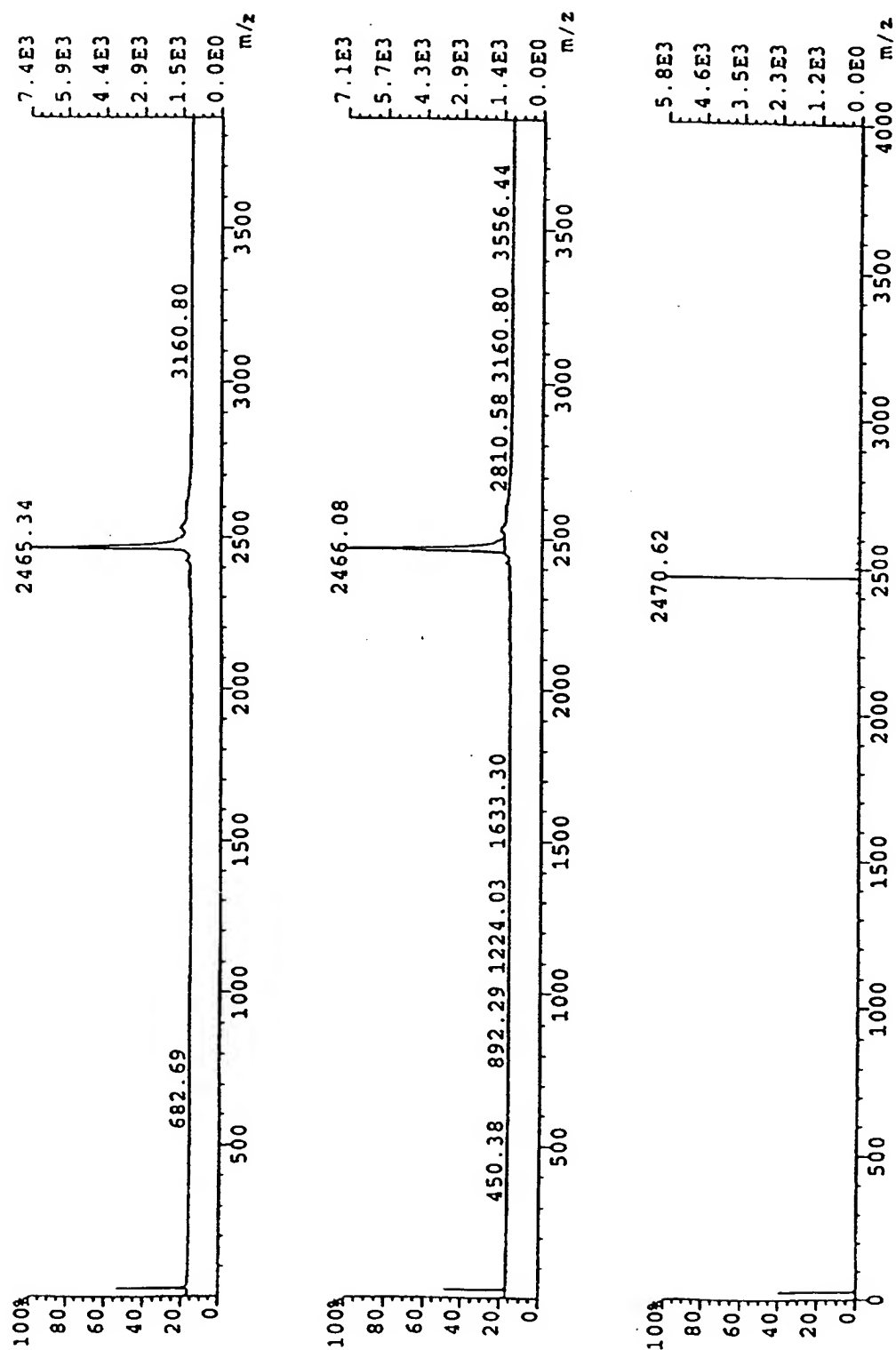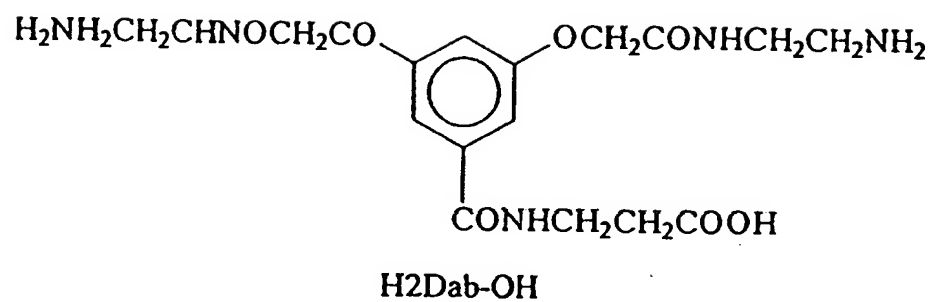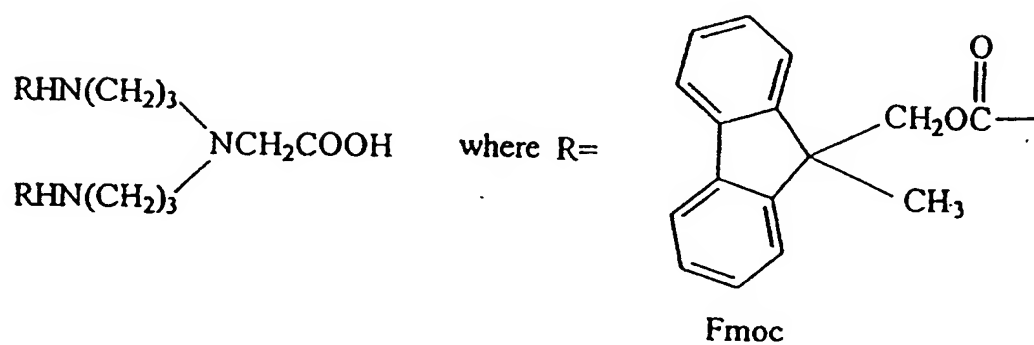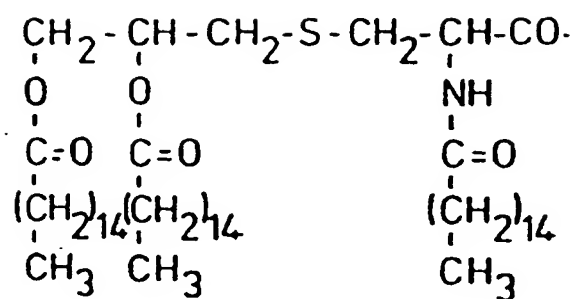
Fig. 4C
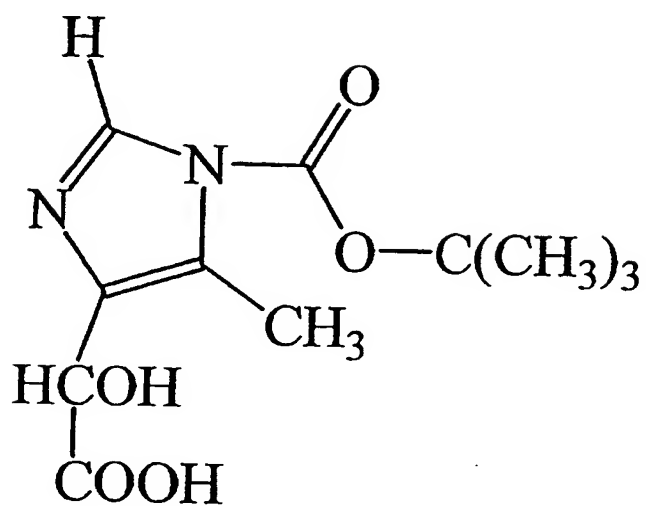
2-[1-*tert*-butoxycarbonyl-5-methylimidazol-4-yl]-2-
hydroxy acetic acid

Fig. 4D
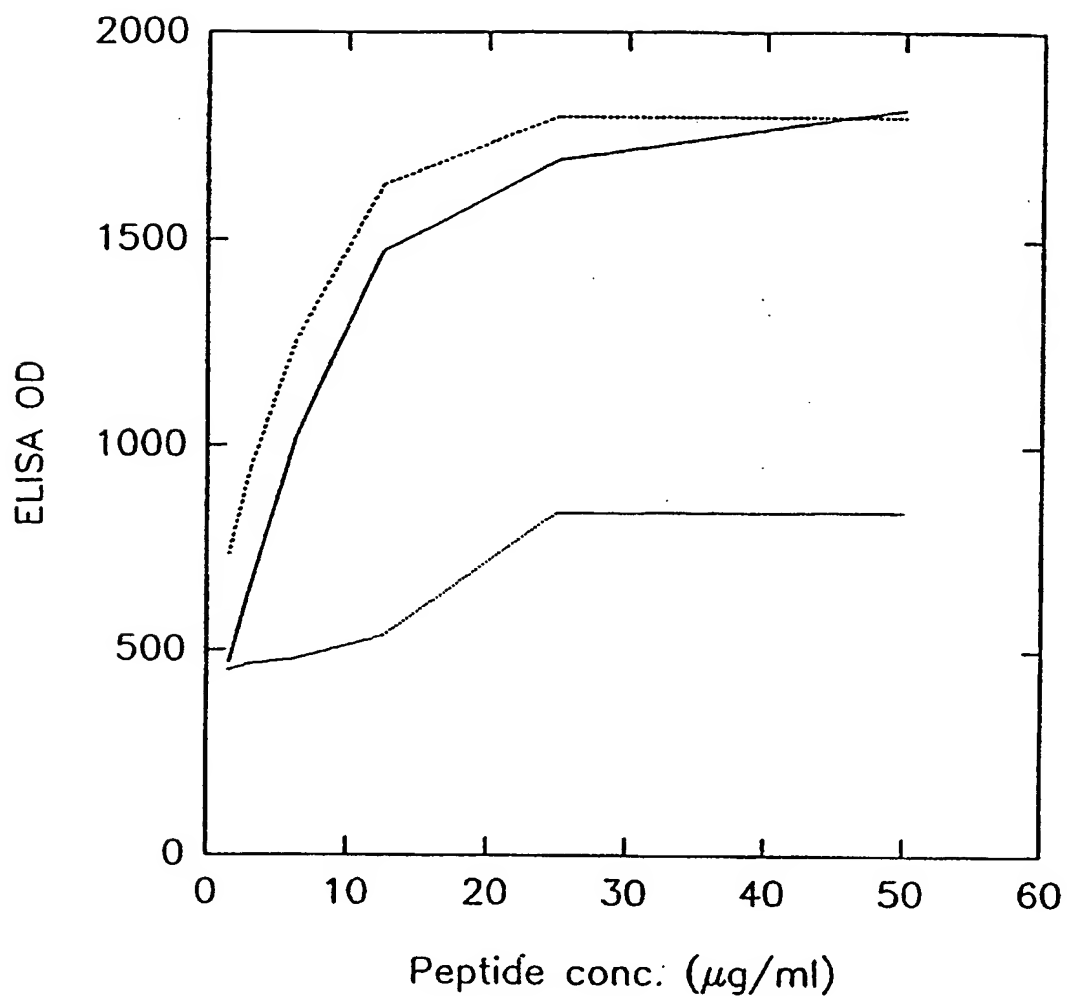
— = Coating of gp41 (aa 598-609)
   linked to carrier at pH 2.5

------ = Coating of gp41 (aa 598-609)
   linked to carrier at pH 9.2

·········· = Coating of gp41 (aa 598-609)
   without carrier at pH 2.5

Fig. 5
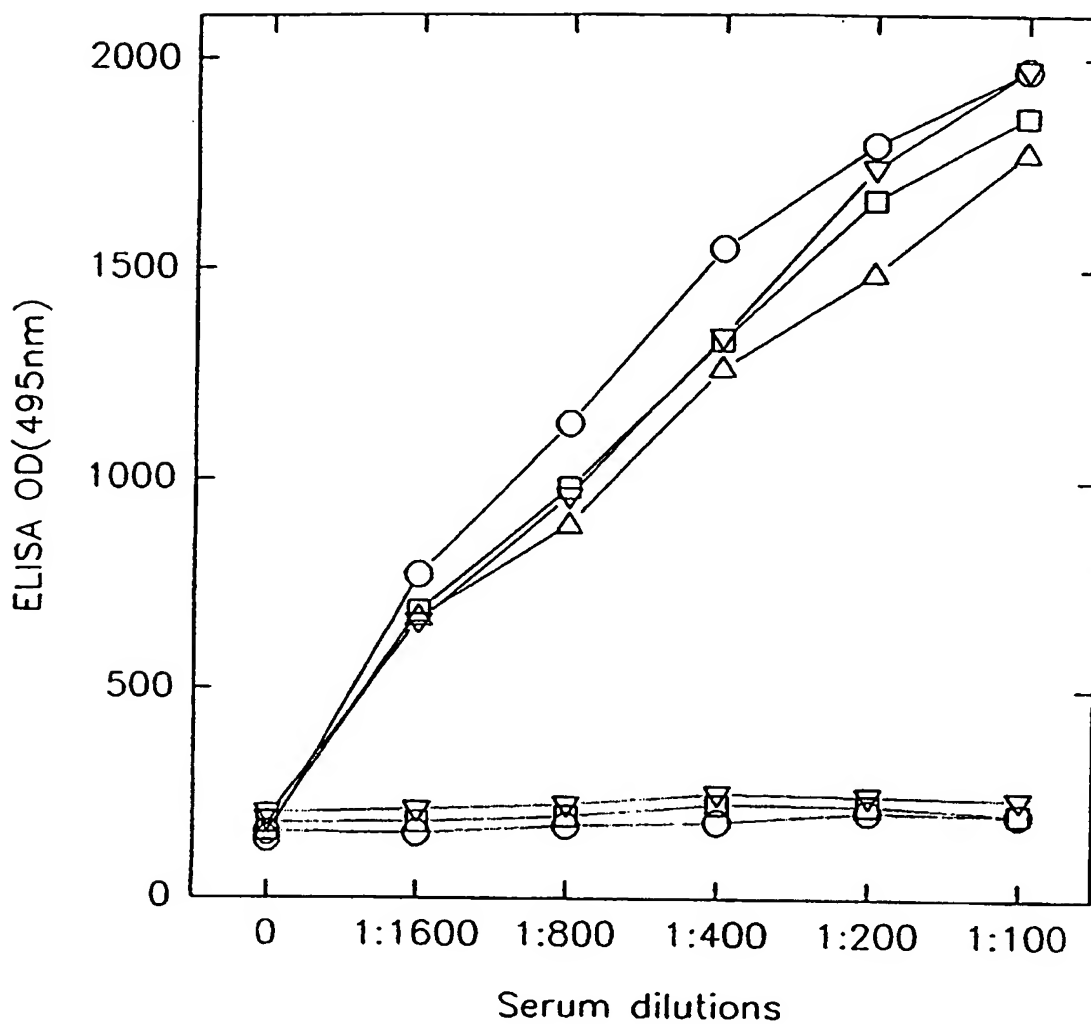
········· = HIV-2 seronegative donors

—— = HIV-2 seropositive donors

Symbols indicating individual donors

Fig. 6
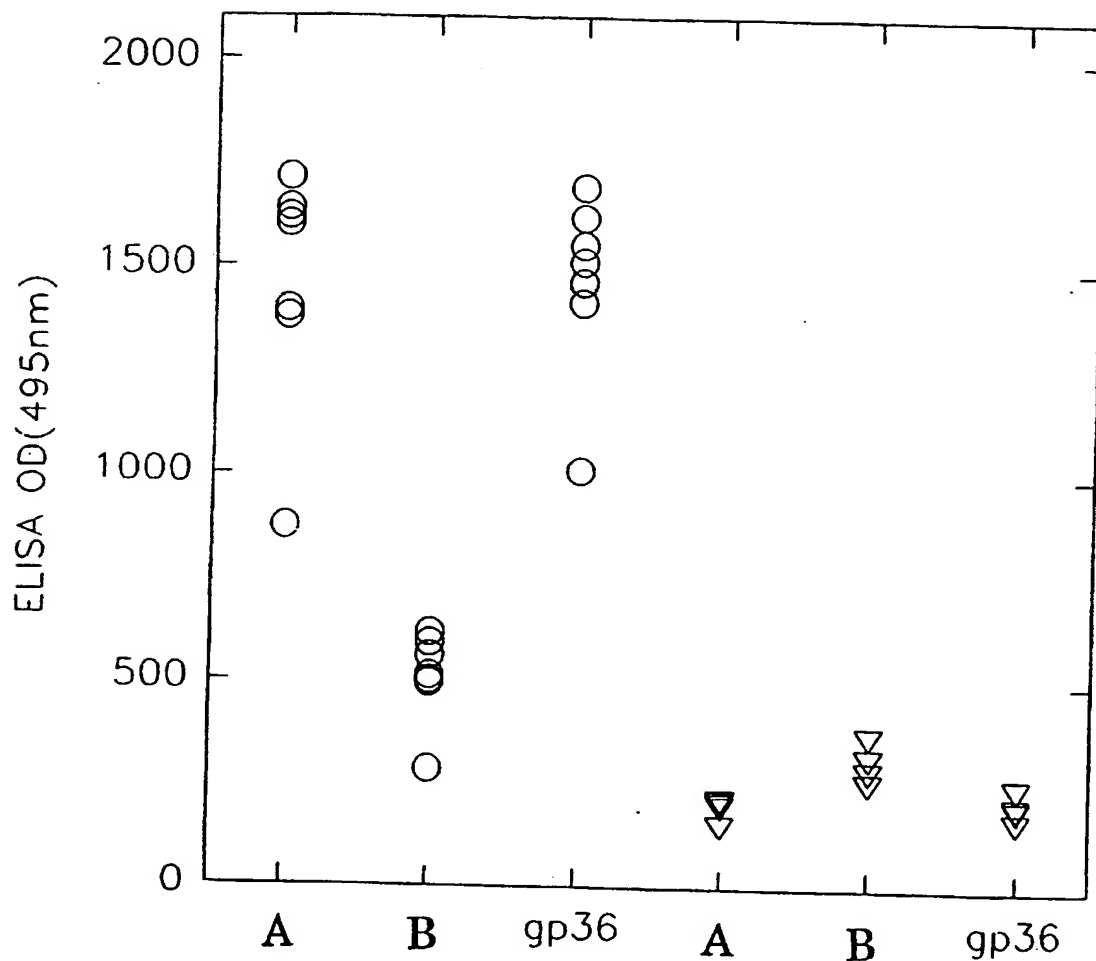
o = HIV-2 seropositive

v = HIV-2 seronegative

A = peptide linked to non-dendritic
    peptide carrier

B = peptide alone

Fig. 7
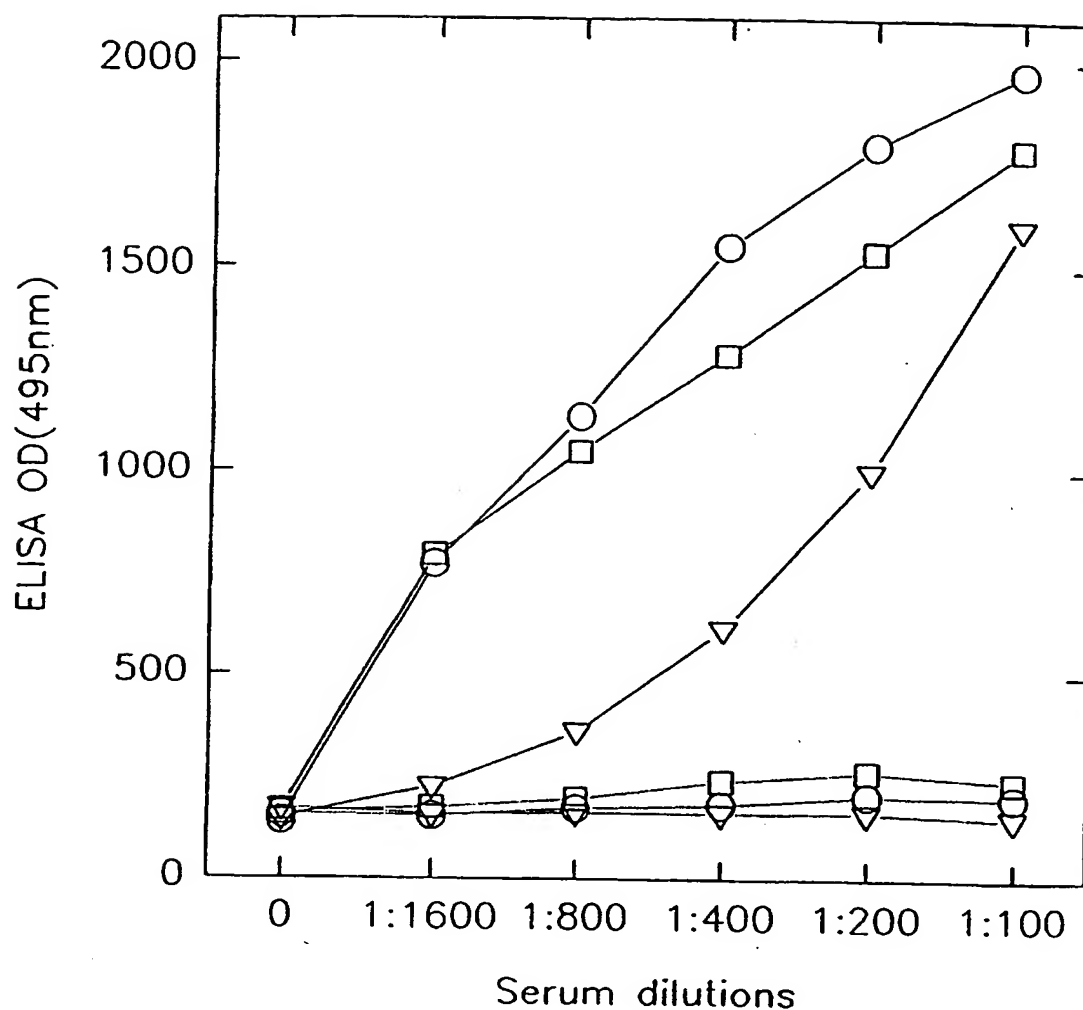
— = HIV-2 seropositive donor

······ = HIV-2 seronegative donor

o = gp36 (aa 587-605) linked to carrier

∇ = gp36 (aa 587-605) not linked to carrier

□ = recombinant gp36

Fig. 8
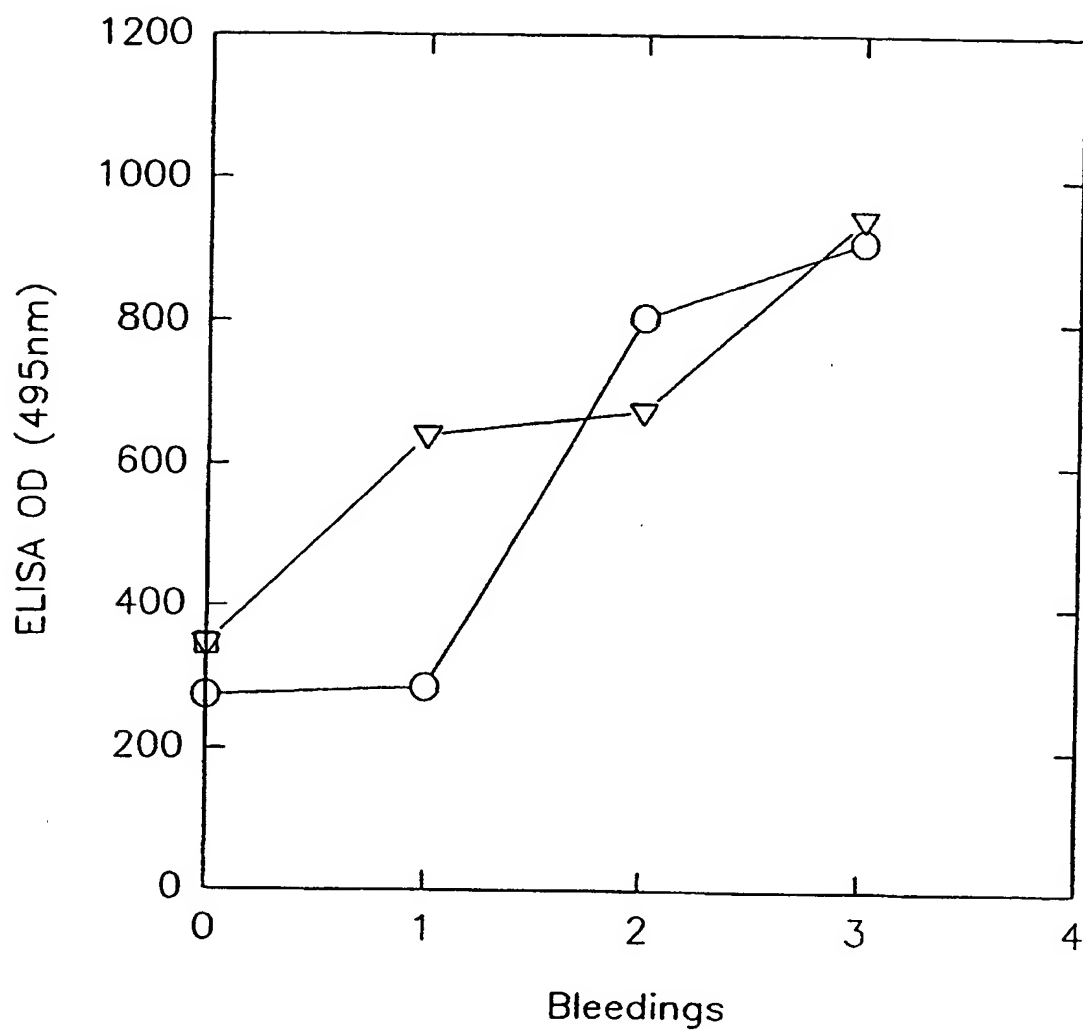
o = without aluminium hydroxide and with PPD
∇ = with aluminium hydroxide and with PPD

Fig. 9
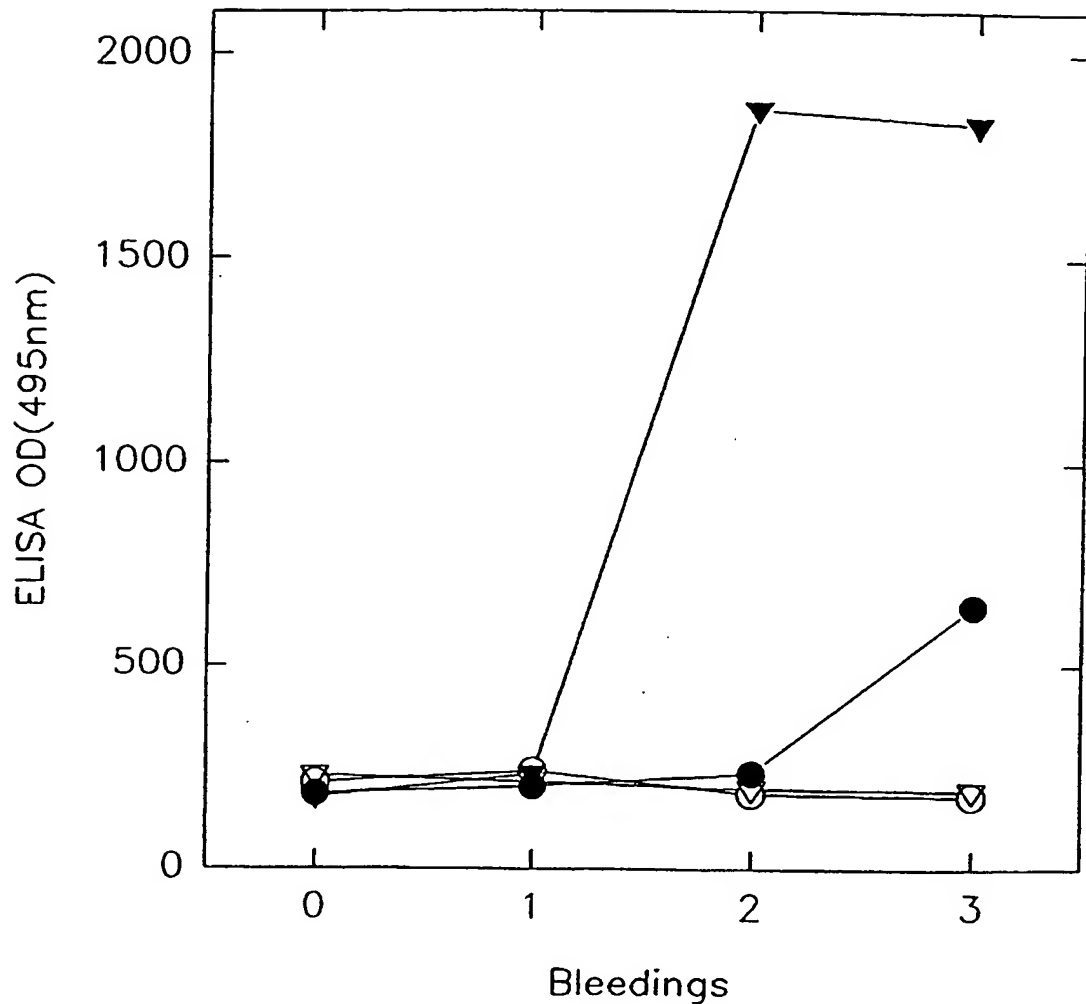
= Immunization i.p. with erythrocyte binding
  peptide linked to carrier

o = As ● but without erythrocyte binding peptide

▼ = Immunization s.c. with erythrocyte binding
  peptide linked to carrier together with
  Freunds adjuvant

▽ = As ▼ but without erythrocyte binding peptide

Fig. 10
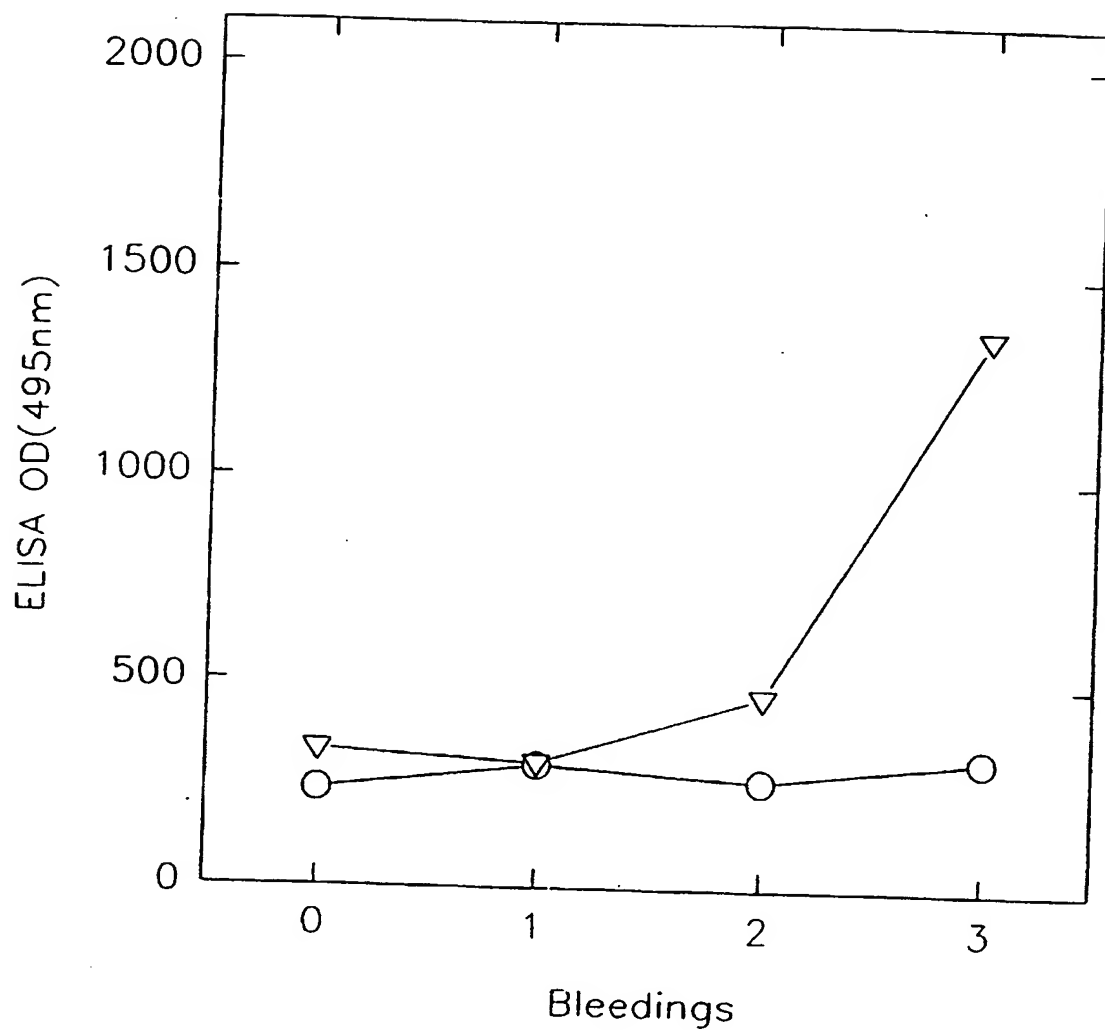
v = Immunization s.c. with gp120 linked
       to carrier and with Freunds adjuvant
o = Immunizaton i.p. with gp120 linked
       to carrier

Fig. 11
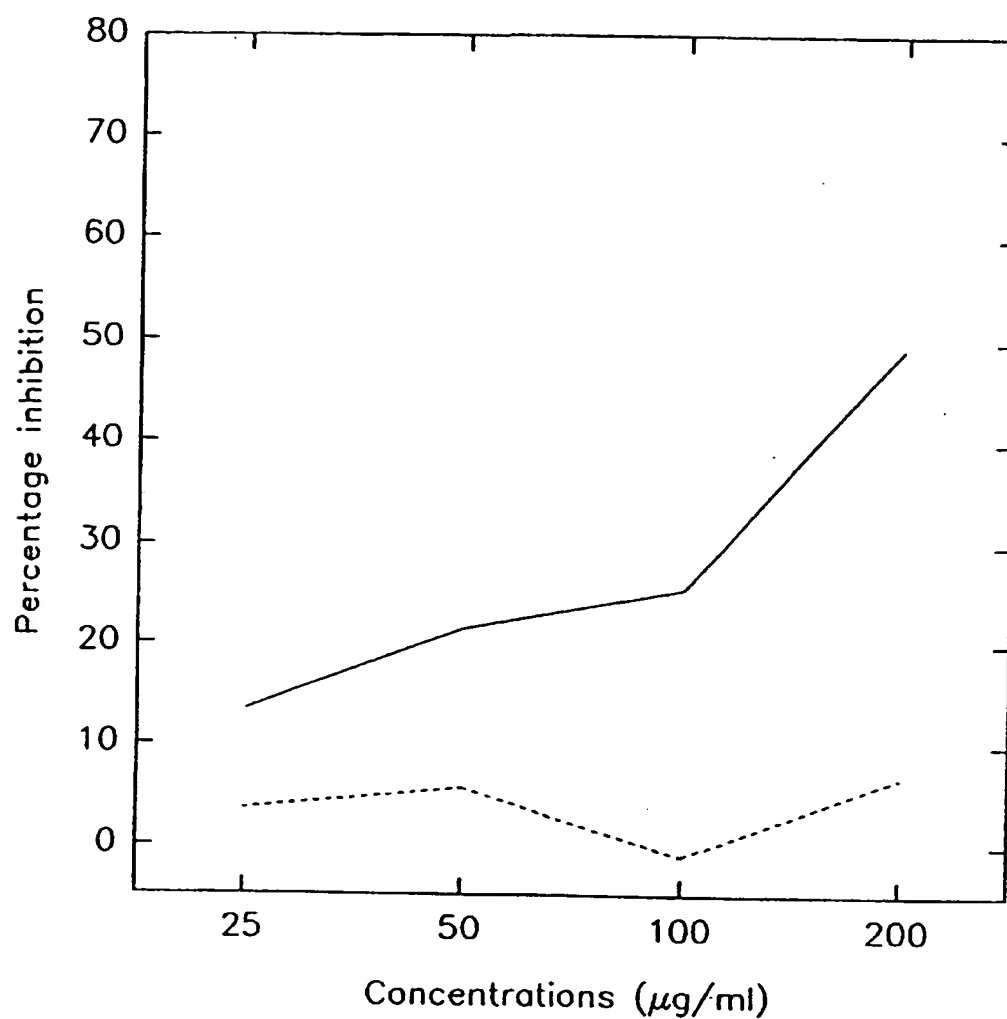
Fig. 12

Fig. 13

Fig. 14

Fig. 15

Fig. 16

IgG reactivity to recombinant gp120

○ Non-dendritic peptide carrier derivatized with gp120 peptide +
Freunds adjuvant s.c.
● Non-dendritic peptide carrier derivatized with gp120 peptide i.p.

Fig. 17

Fig. 18

IgG1 reactivity to gp120 peptide

Bleedings

O △ s.c.
● ▲ i.p.
O ● Non-dendritic peptide carrier derivatized with gp120 peptide
△ ▲ Non-dendritic peptide carrier derivatized with gp120 peptide +
      non-dendritic peptide carrier derivatized with gp41 peptide
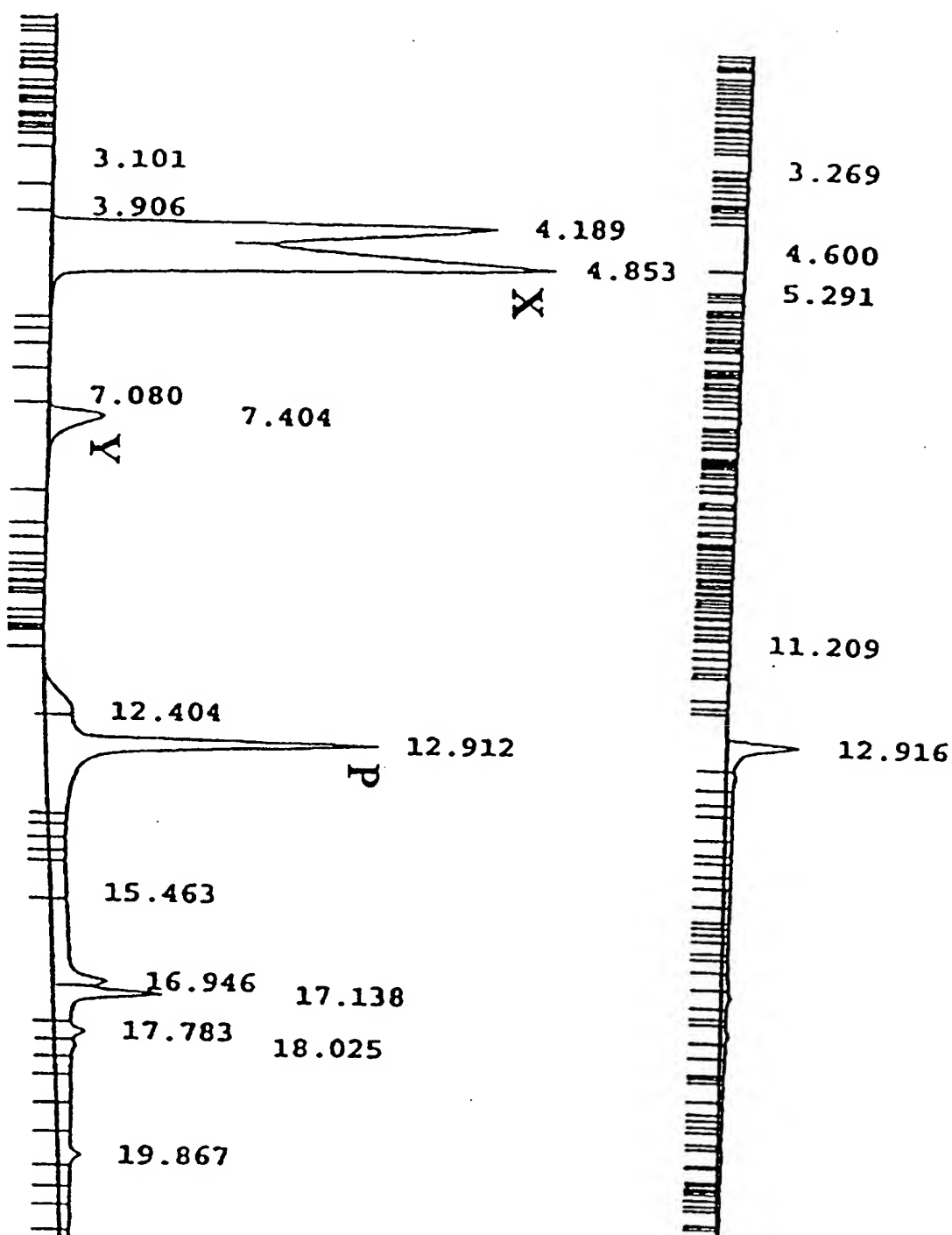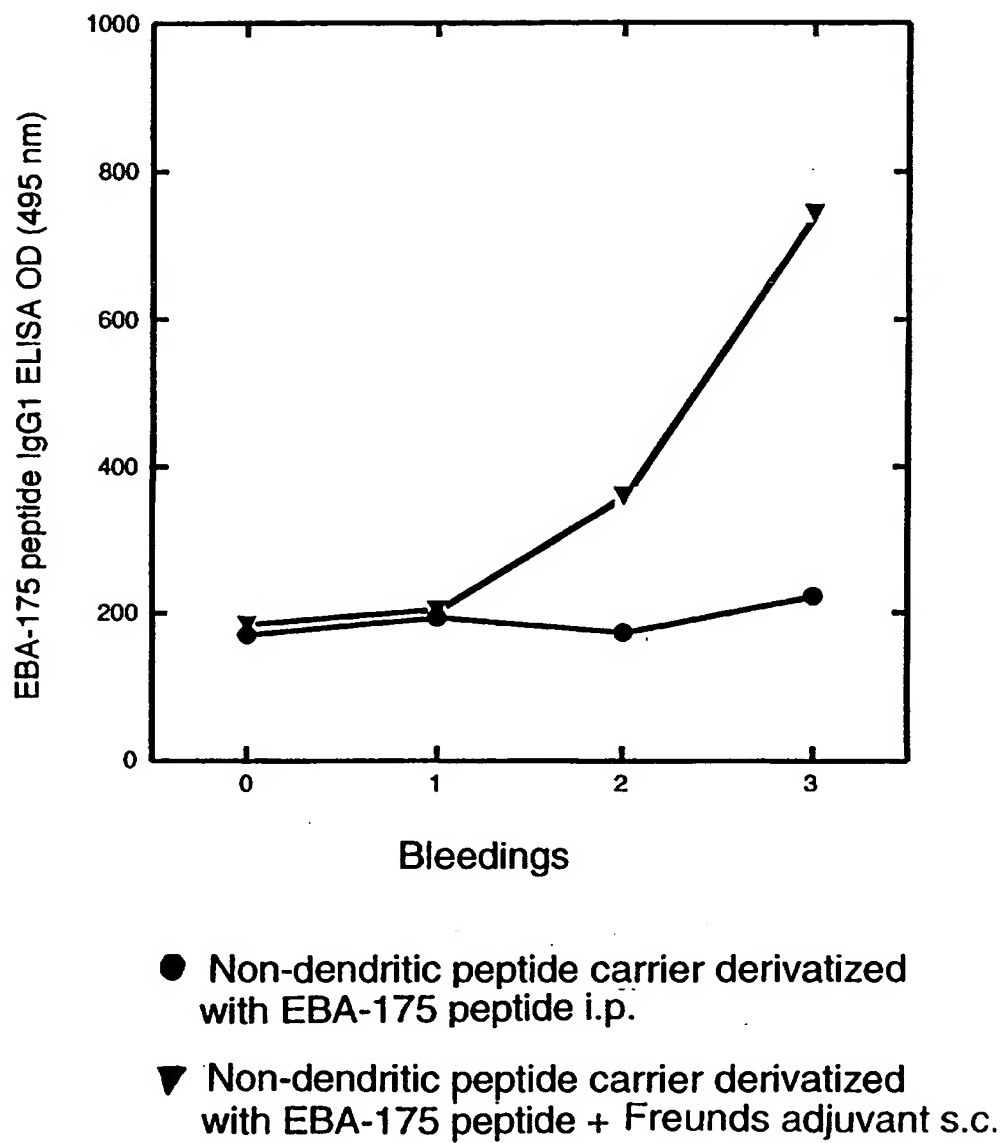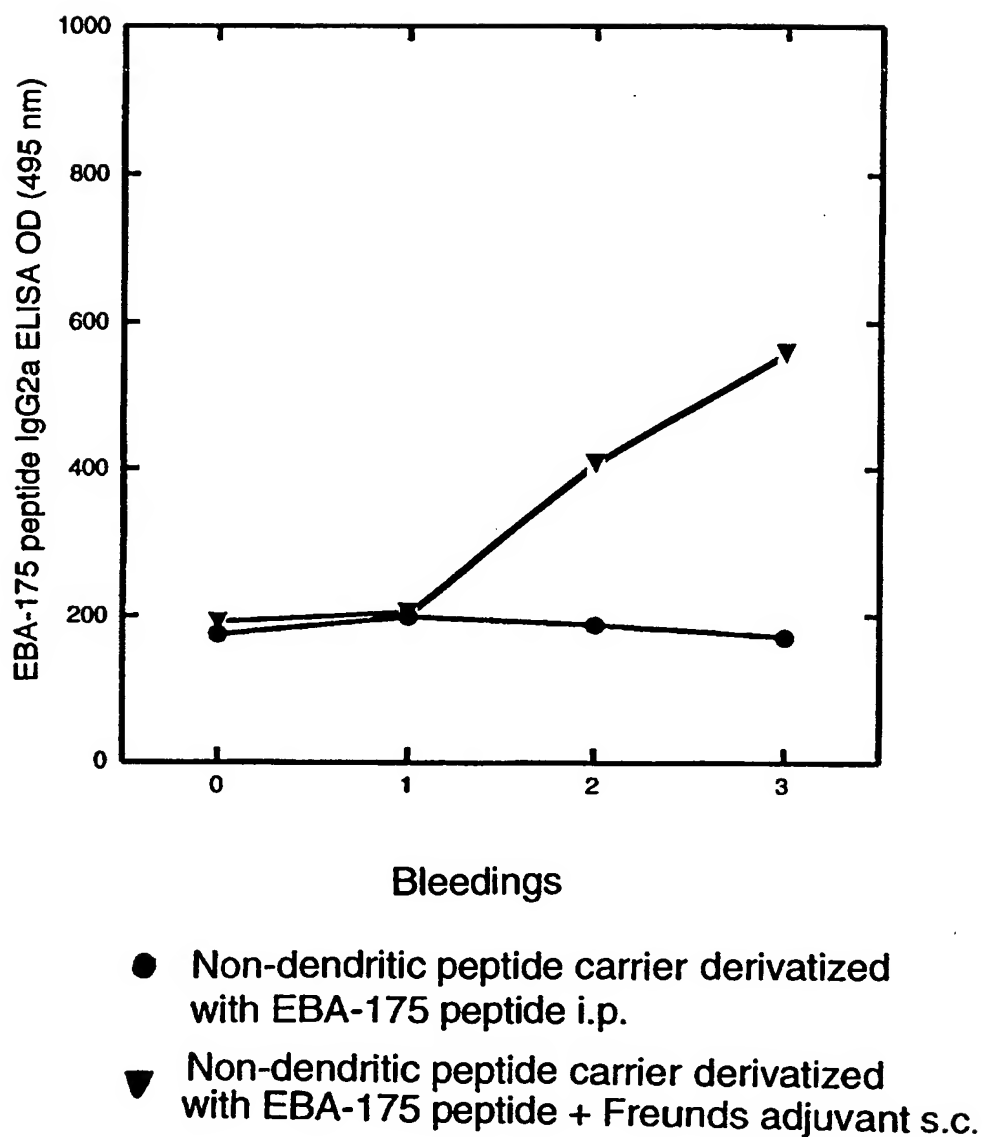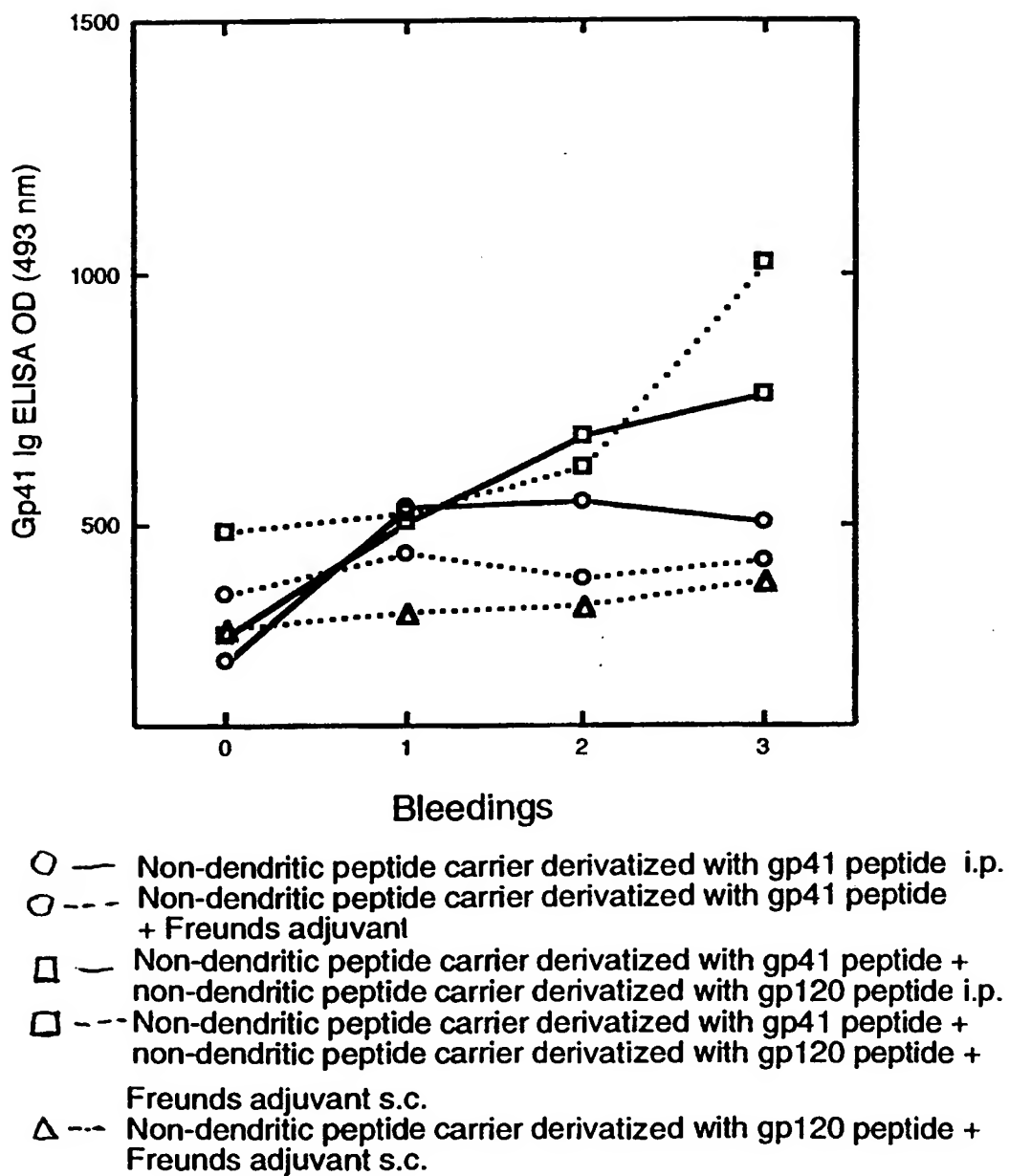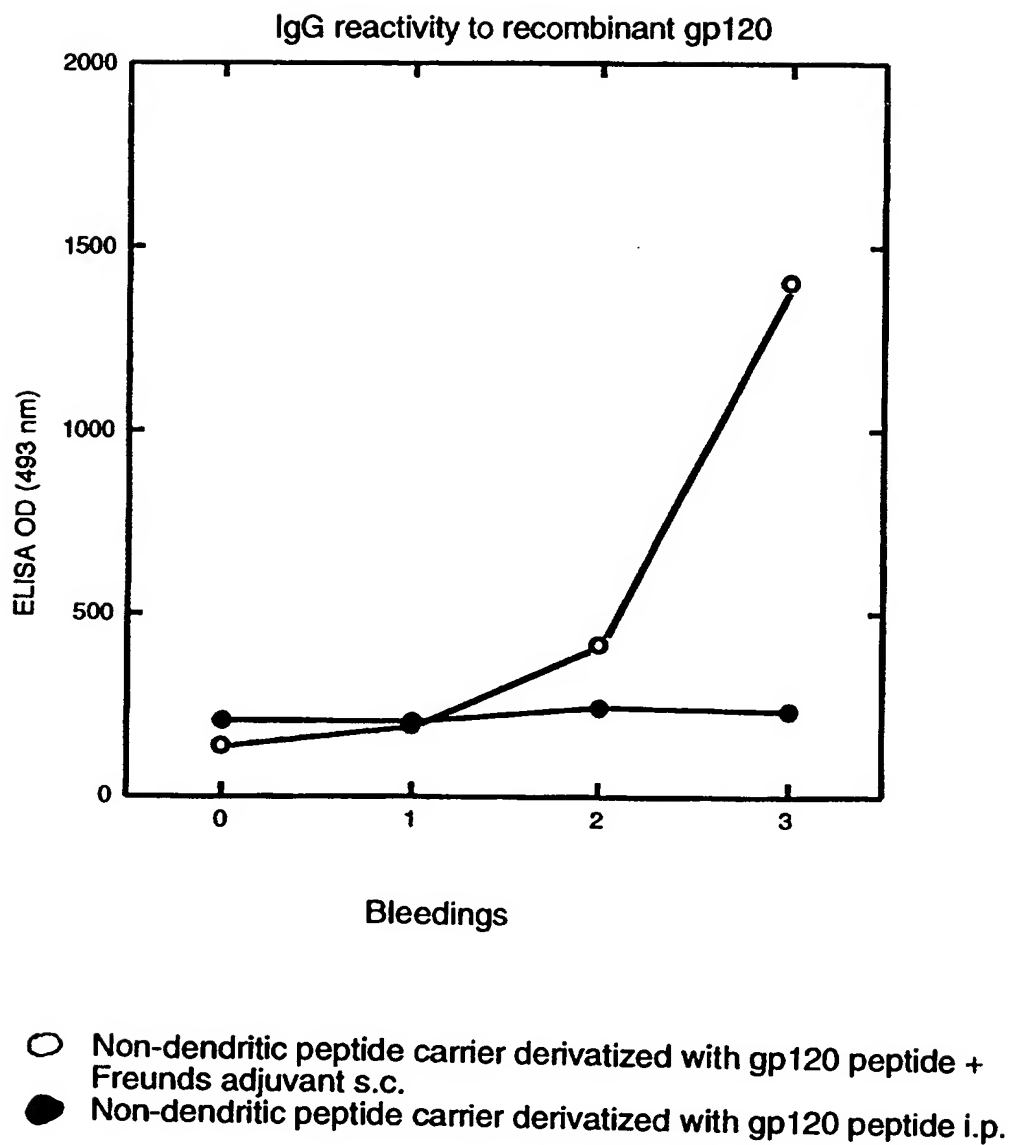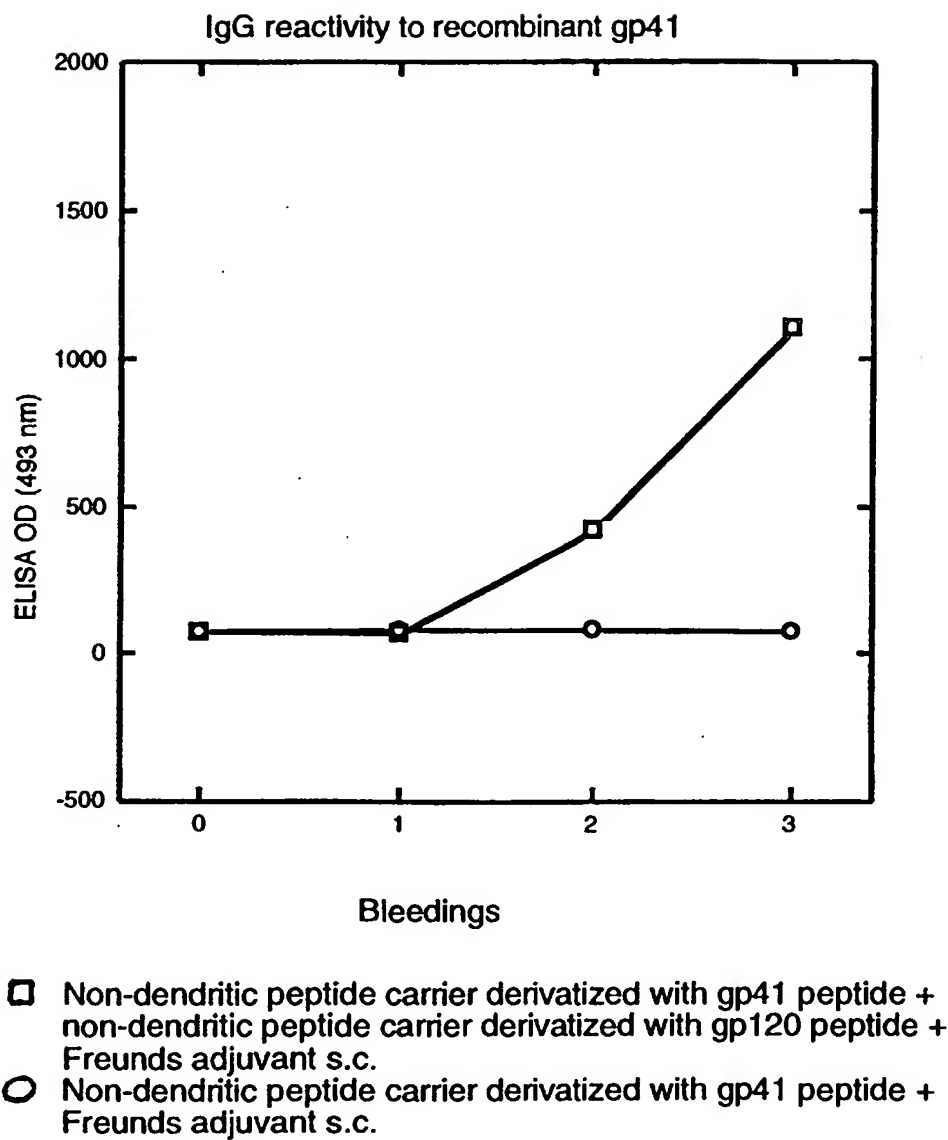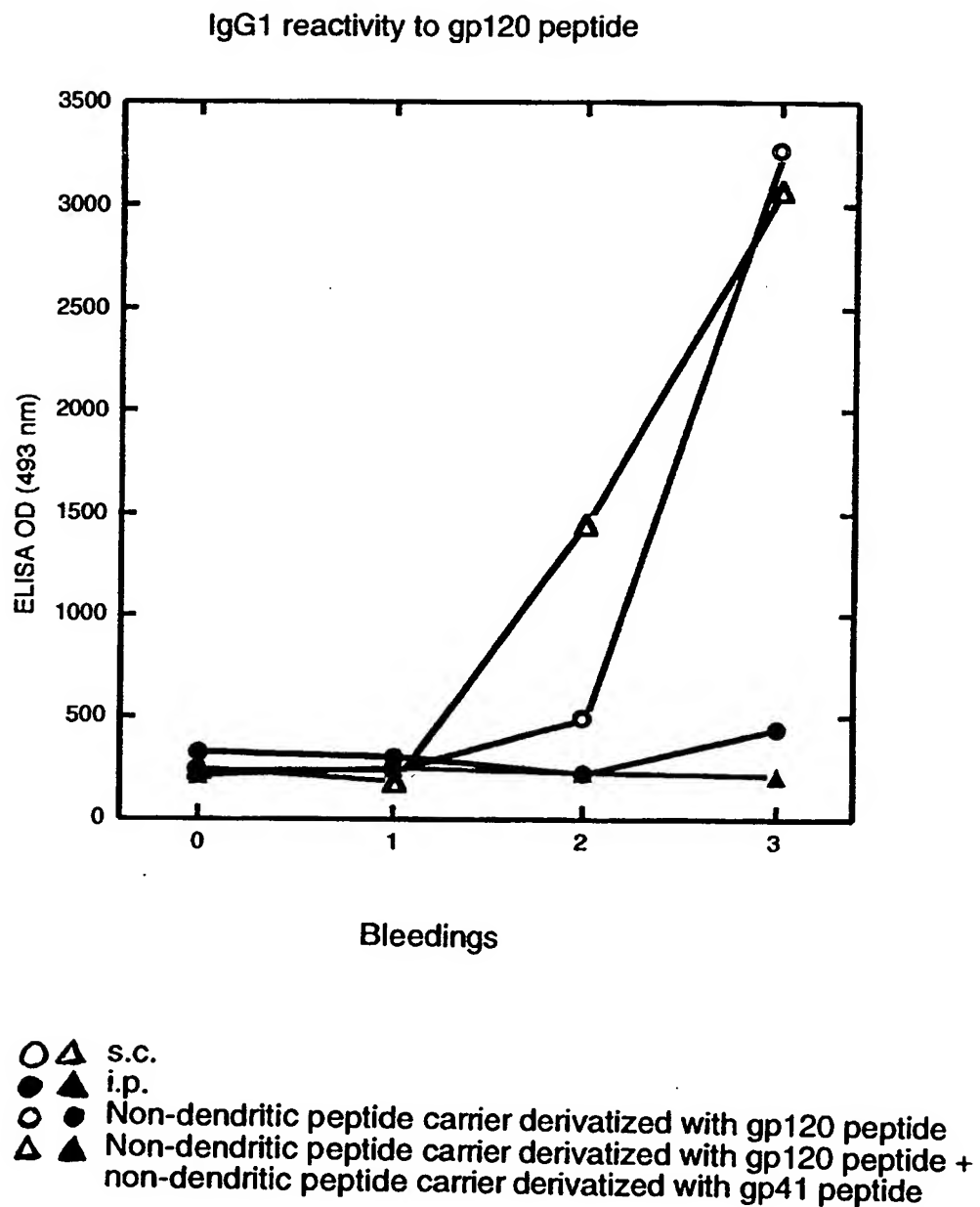
Fig. 19

IgG2a reactivity to gp120 peptide

Fig. 20
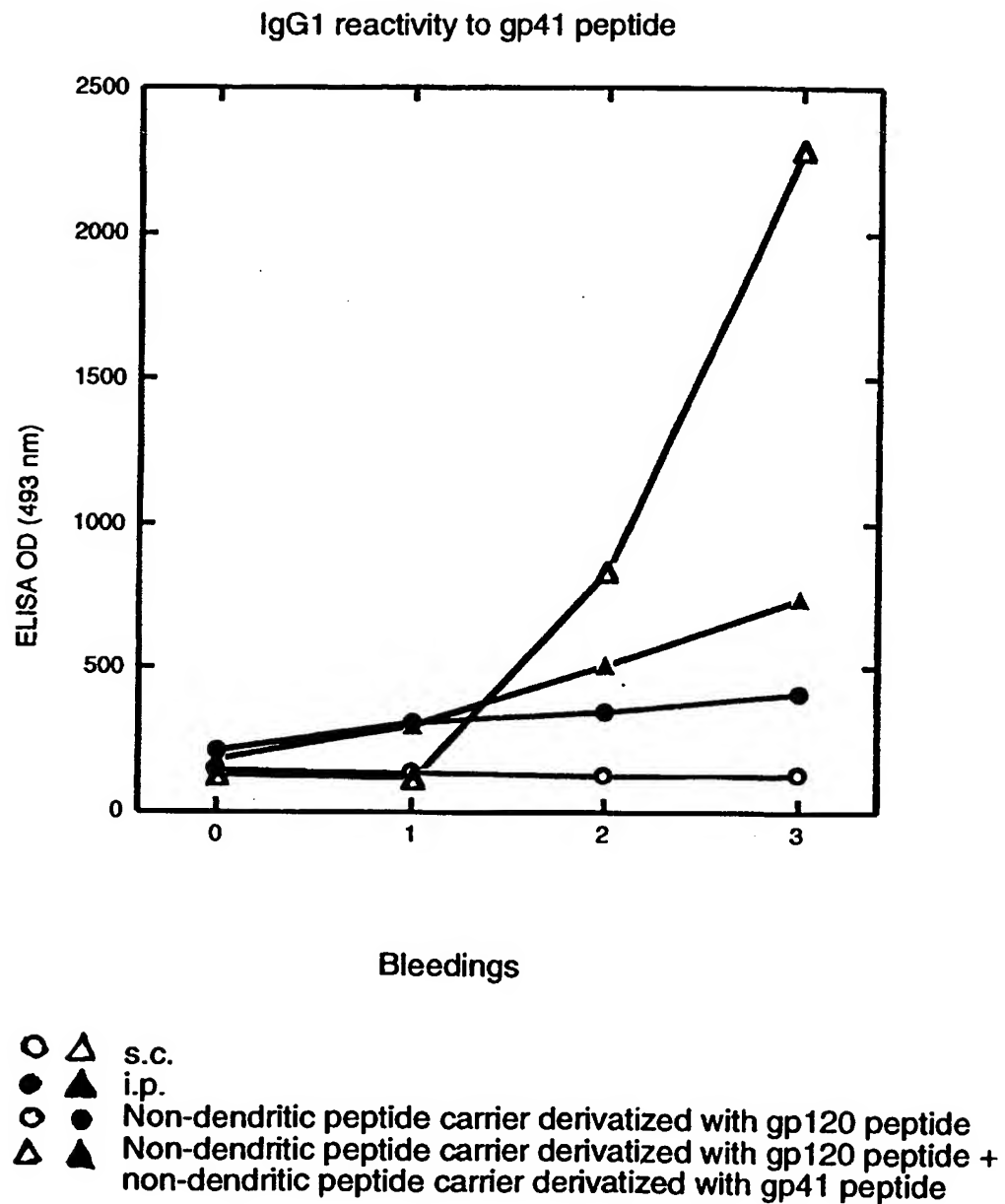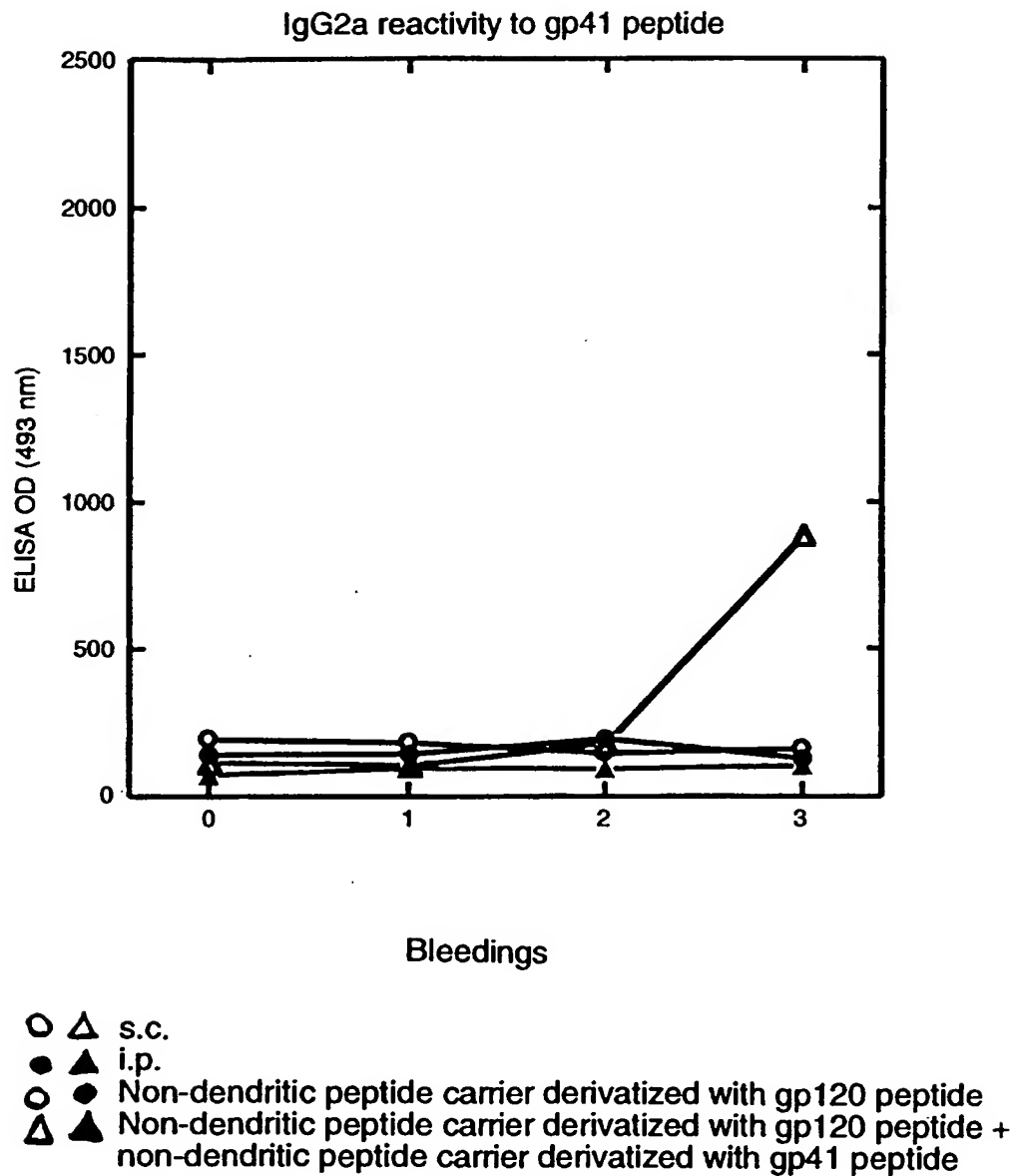
Fig. 21

Fig. 22

Fig. 23

O LPS
☐ Non-dendritic peptide carrier derivatized with CKNKEKKC
    peptide + LPS
△ CKNKEKKC peptide + LPS
▽ Non-dendritic peptide carrier derivatized with KNGMLKGDKVS
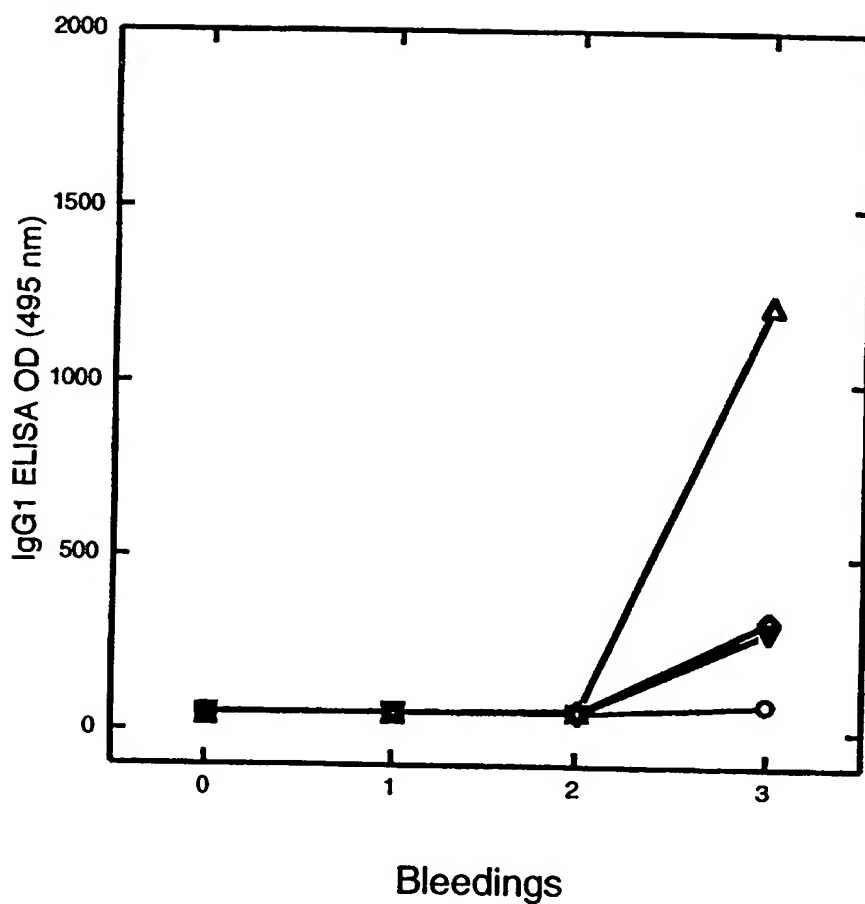   peptide + LPS
• Non-dendritic peptide carrier + LPS
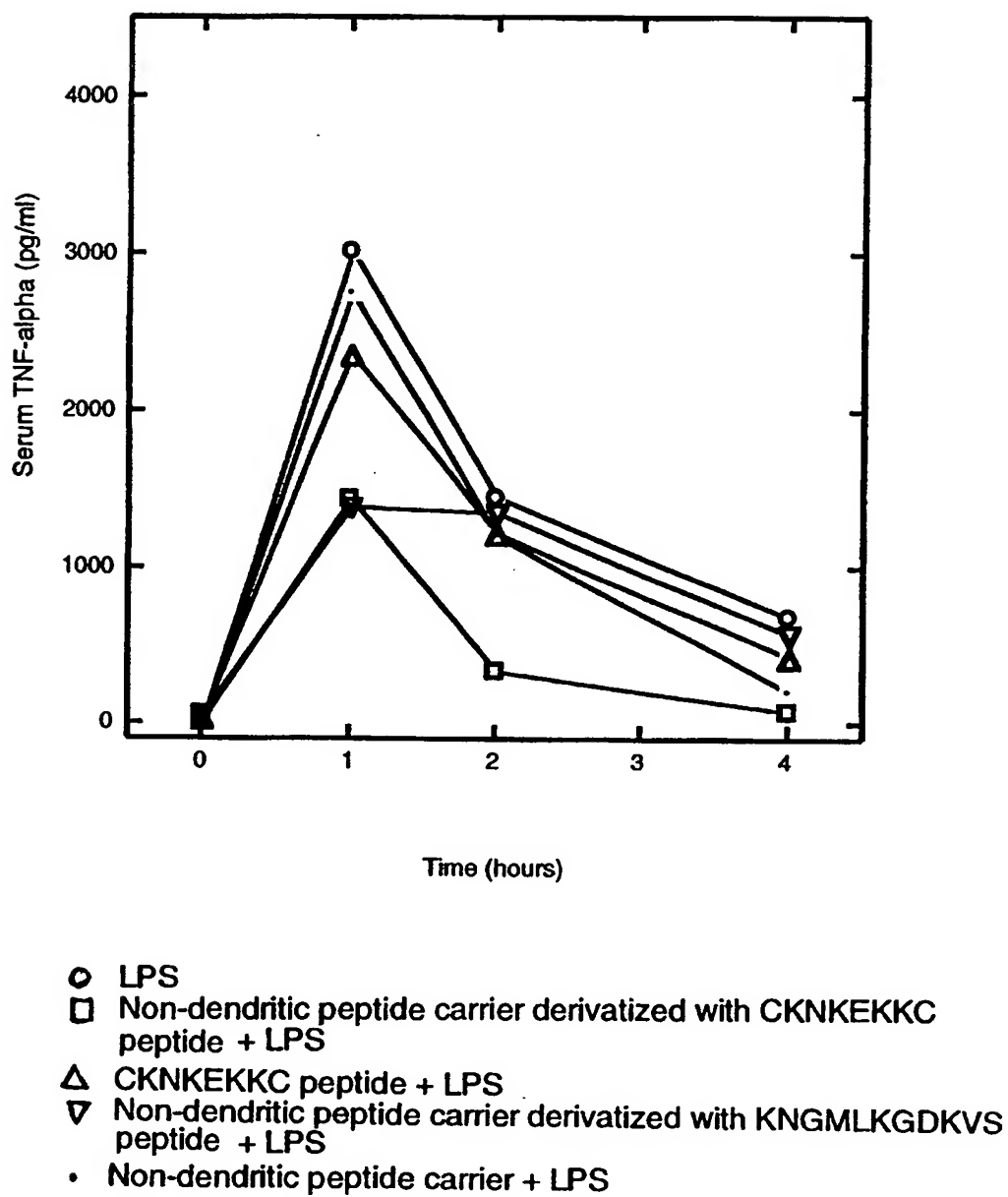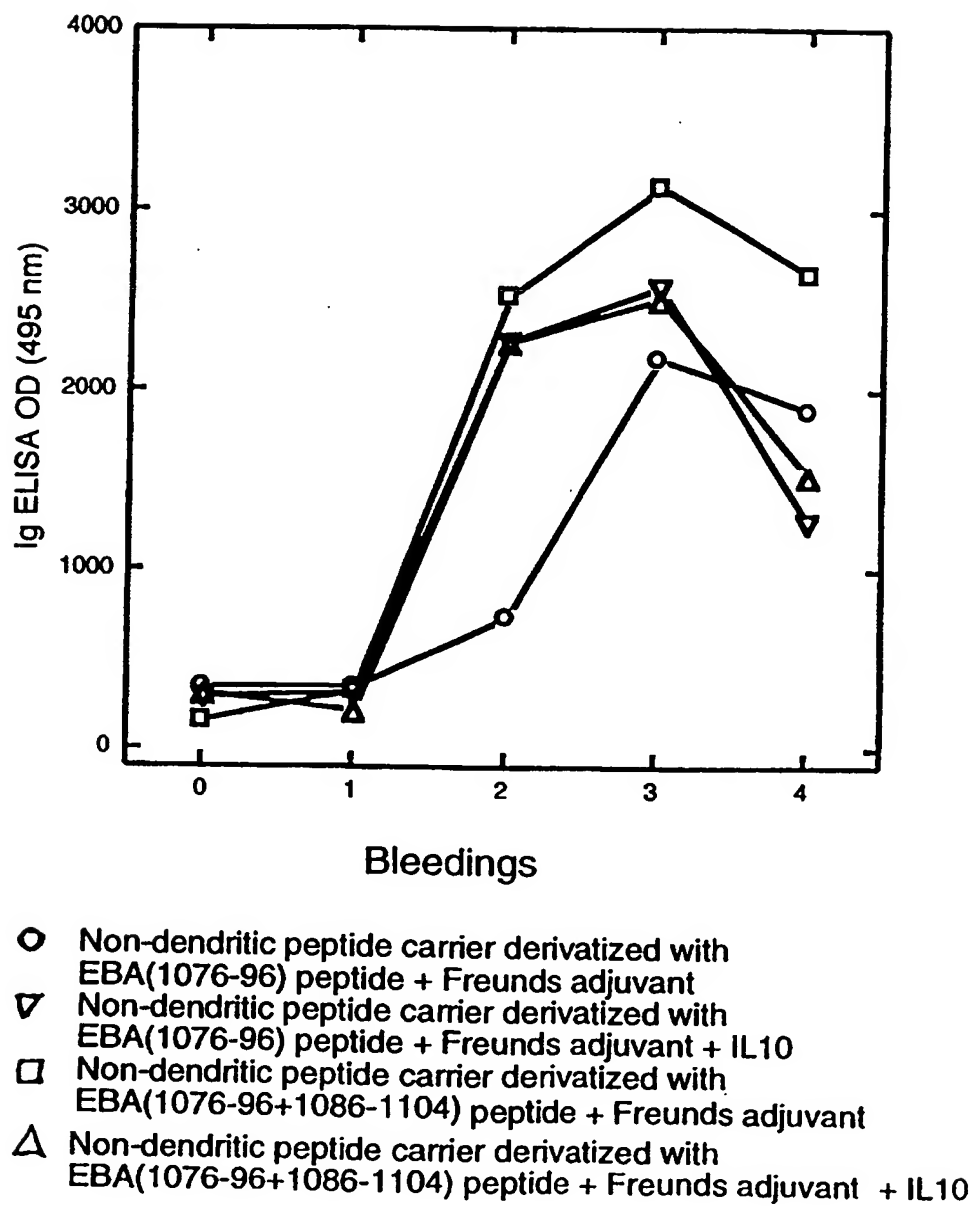
Fig. 24
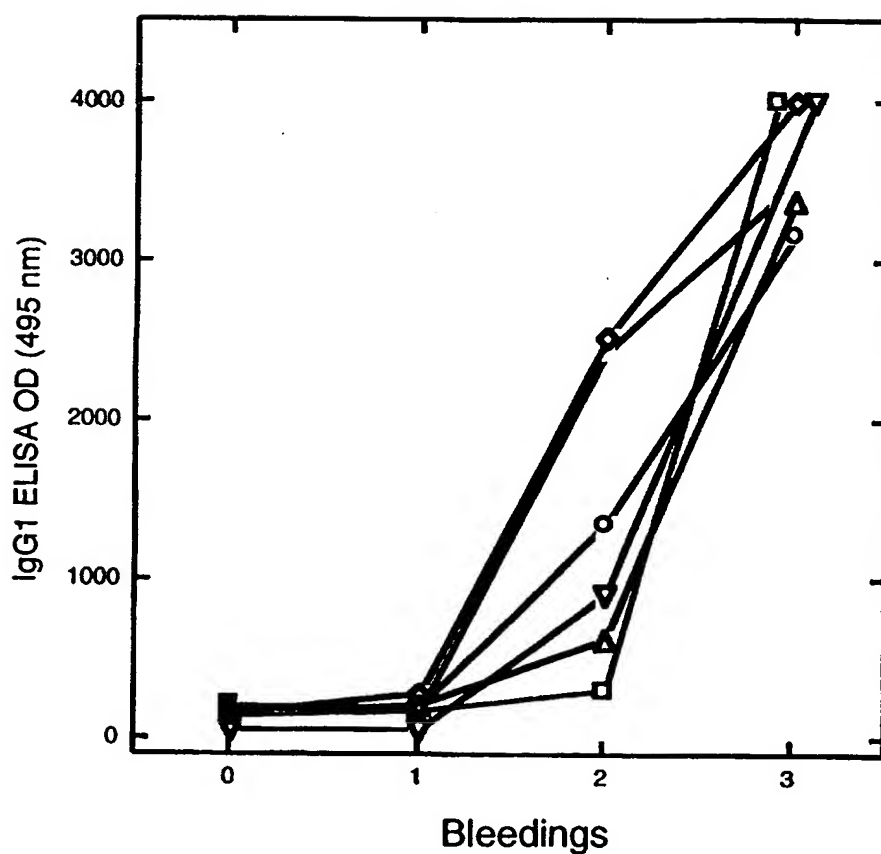
O Non-dendritic peptide carrier derivatized with L1 peptide+alumn s.c.
□ Non-dendritic peptide carrier derivatized with L1 peptide + Freunds
   complete adjuvant s.c.
▽ Non-dendritic peptide carrier derivatized with L1 peptide + rek.
   IFNgamma + alumn s.c.
• Non-dendritic peptide carrier derivatized with L1 peptide +
   rek. IL-12 + alumn s.c.
◇ Non-dendritic peptide carrier derivatized with L1 peptide +
   rek. TNF + alumn s.c.
△ Non-dendritic peptide carrier derivatized with L1 peptide +
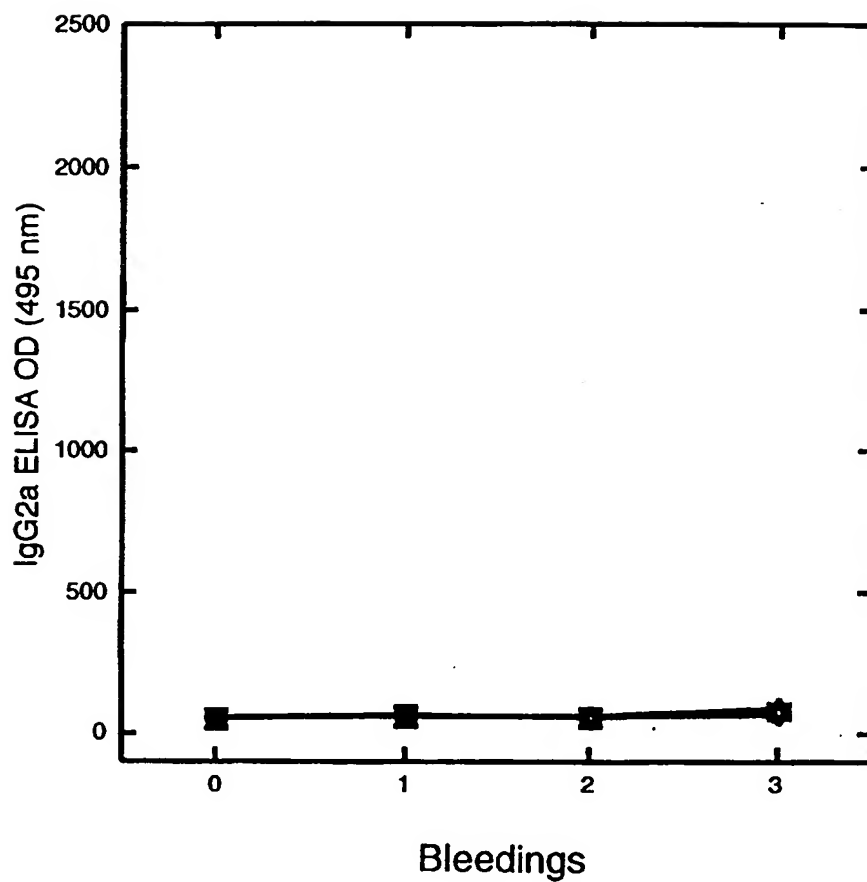   rek. IL-4 + alumn s.c.

Fig. 25

Fig. 26

Fig. 27

Fig. 28

Fig. 29
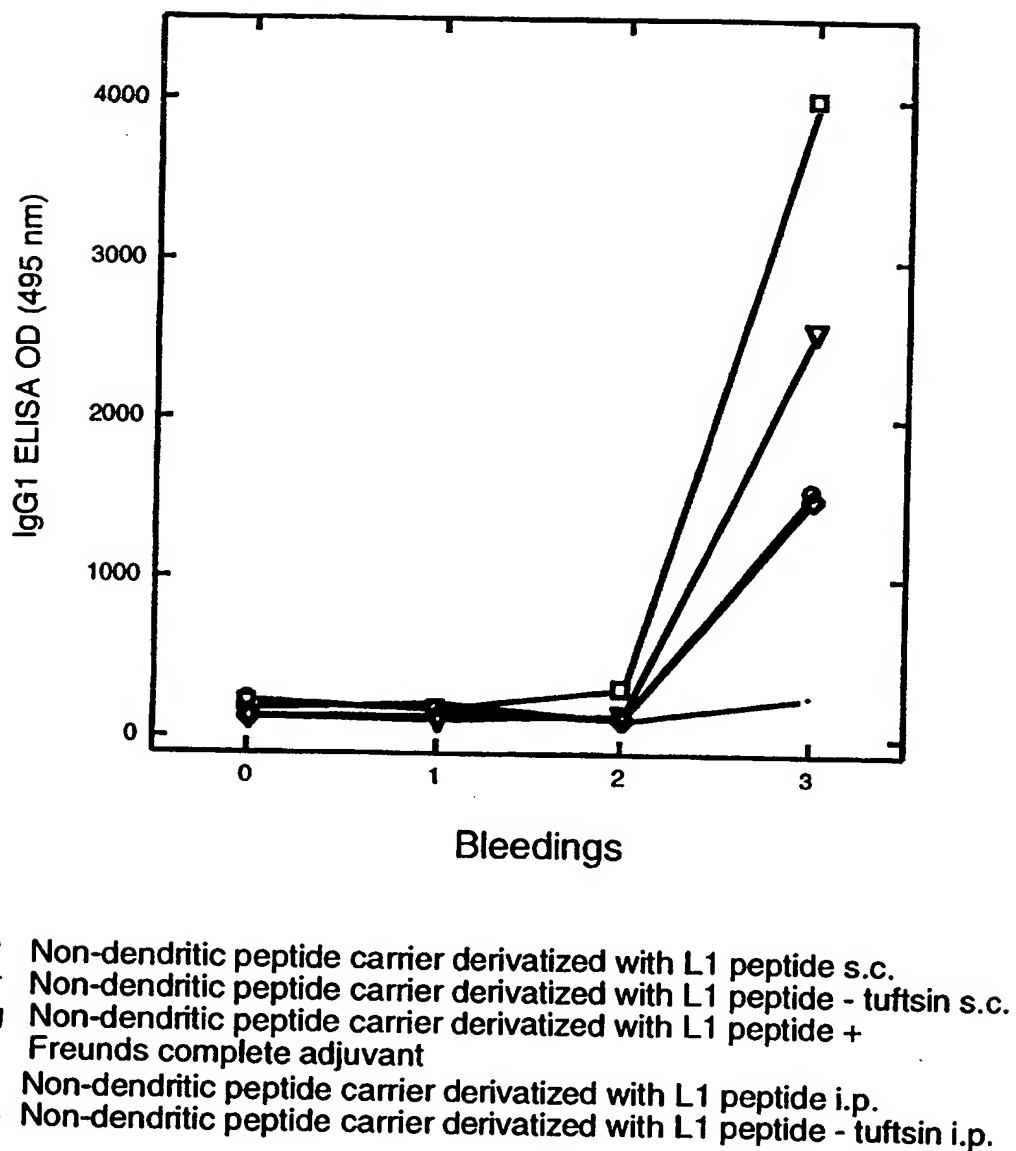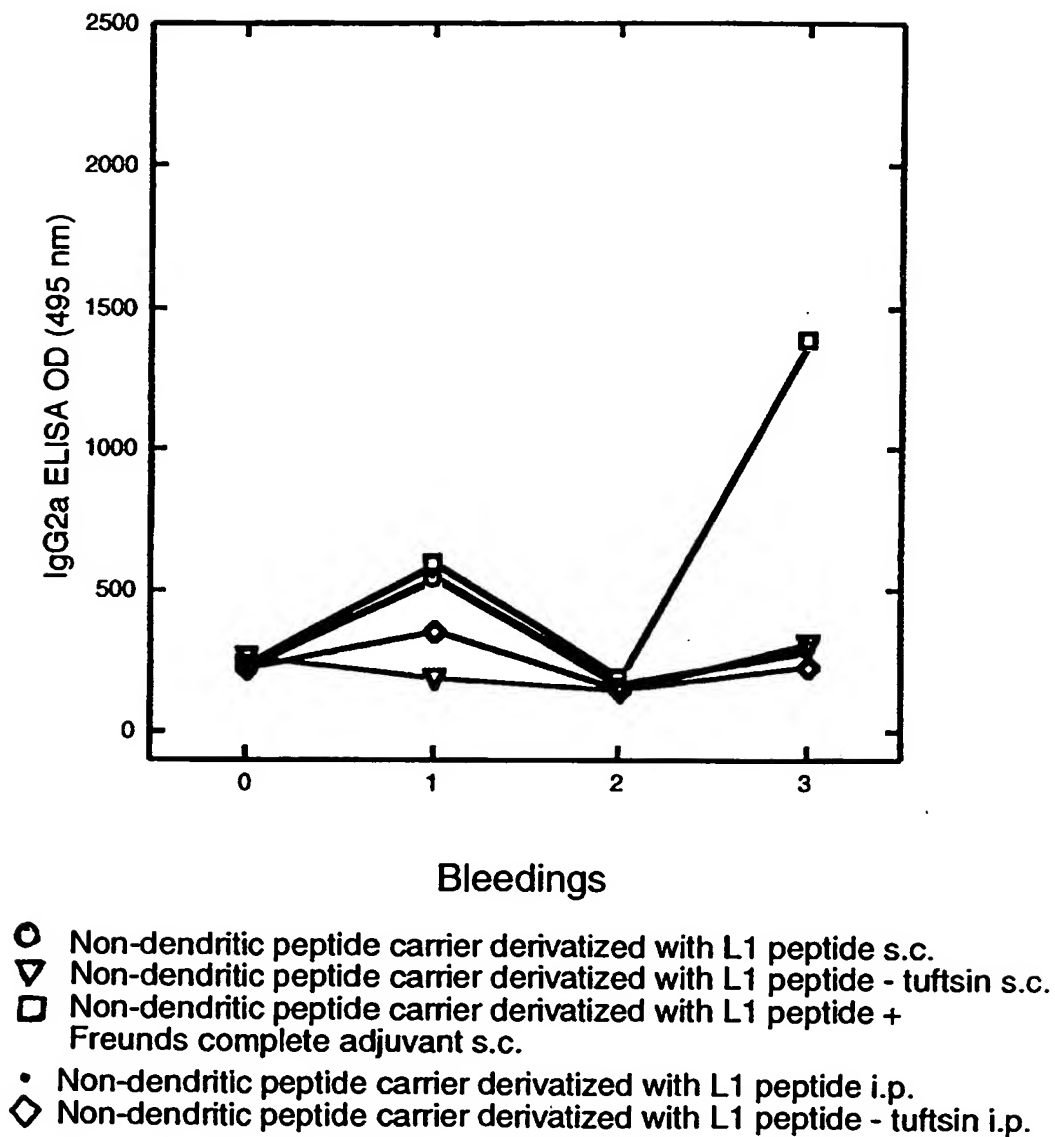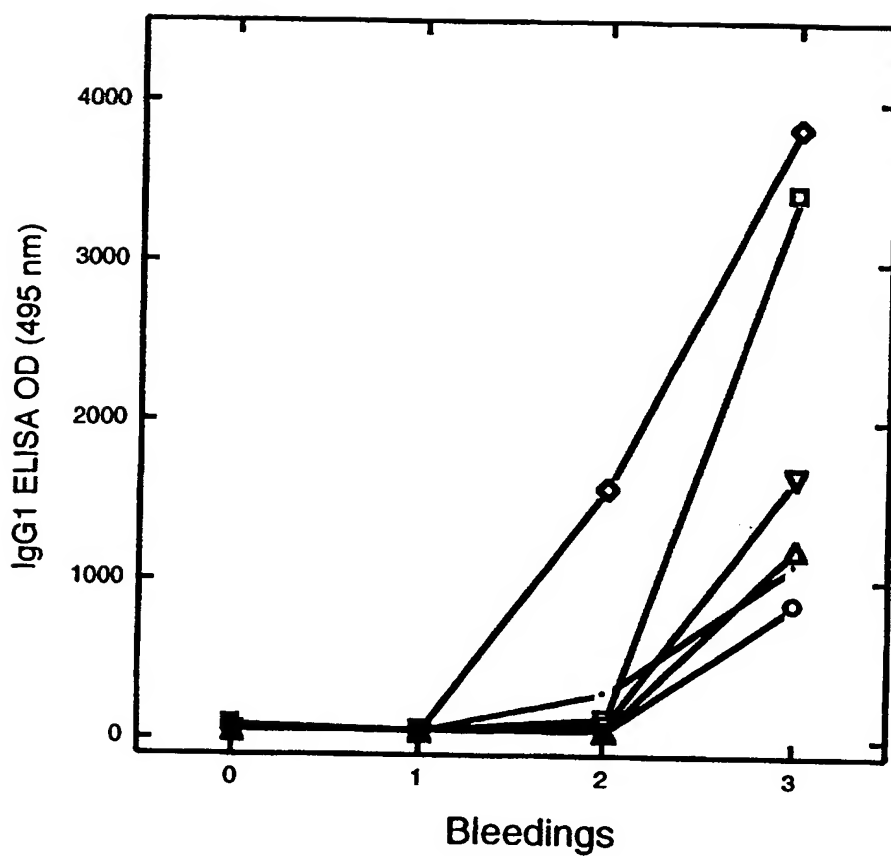
O Non-dendritic peptide carrier derivatized with L1 peptide s.c.
□ Non-dendritic peptide carrier derivatized with L1 peptide +
   Freunds adjuvant s.c.
• Non-dendritic peptide carrier derivatized with L1 peptide +
   recombinant interferon-gamma s.c.
▽ Non-dendritic peptide carrier derivatized with
   L1 - IFN(1-39) peptide s.c.
△ Non-dendritic peptide carrier derivatized with
   L1 - IFN(95-133) peptide s.c.
◇ Non-dendritic peptide carrier derivatized with
   L1 - IFN(1-39) + L1 - IFN(95-133) peptide s.c.

Fig. 30

O   Non-dendritic peptide carrier derivatized with L1 peptide i.p.
▽   Non-dendritic peptide carrier derivatized with
    L1 - IFN(1-39) peptide i.p.
△   Non-dendritic peptide carrier derivatized with
    L1 - IFN(95-133) peptide i.p.
◇   Non-dendritic peptide carrier derivatized with
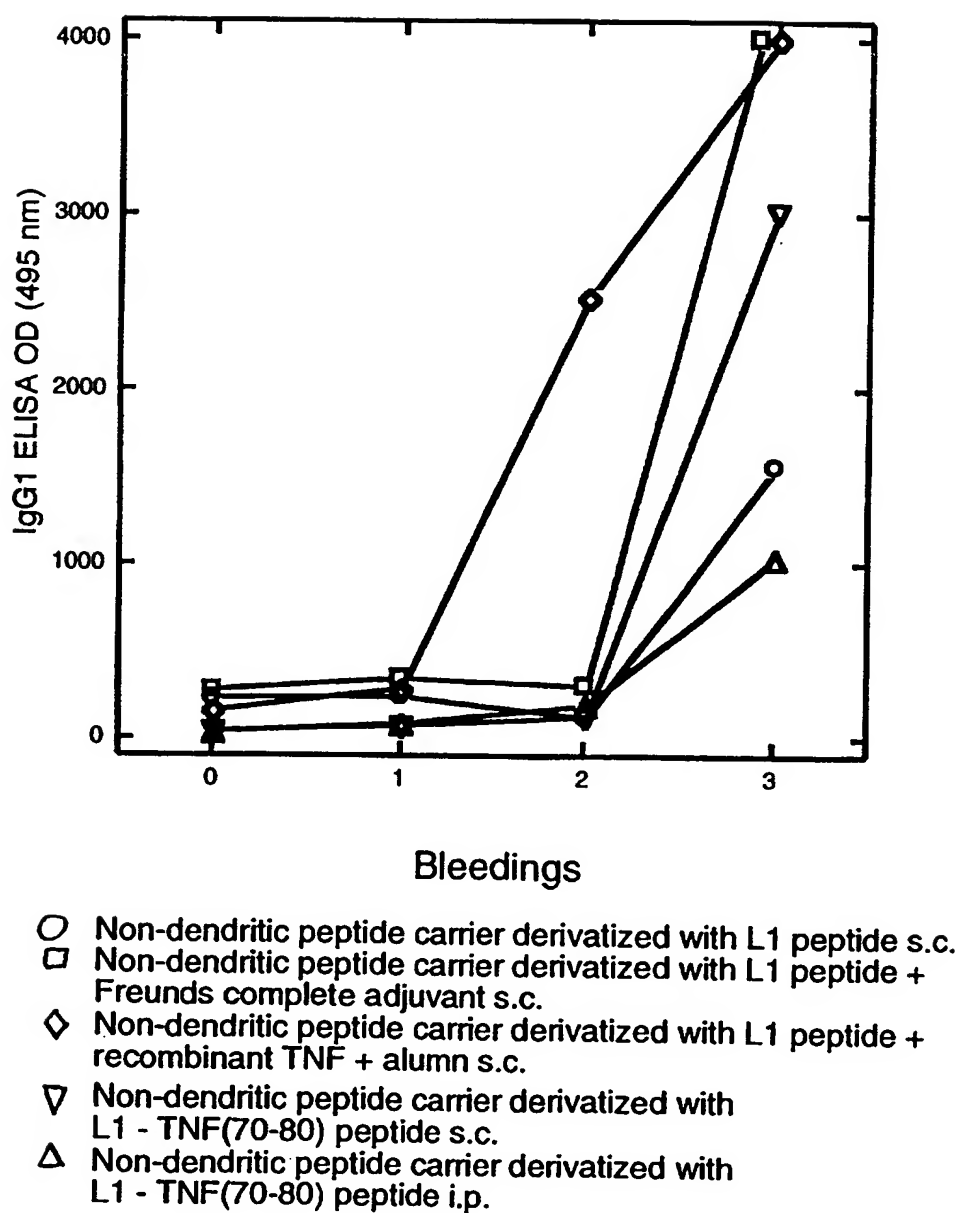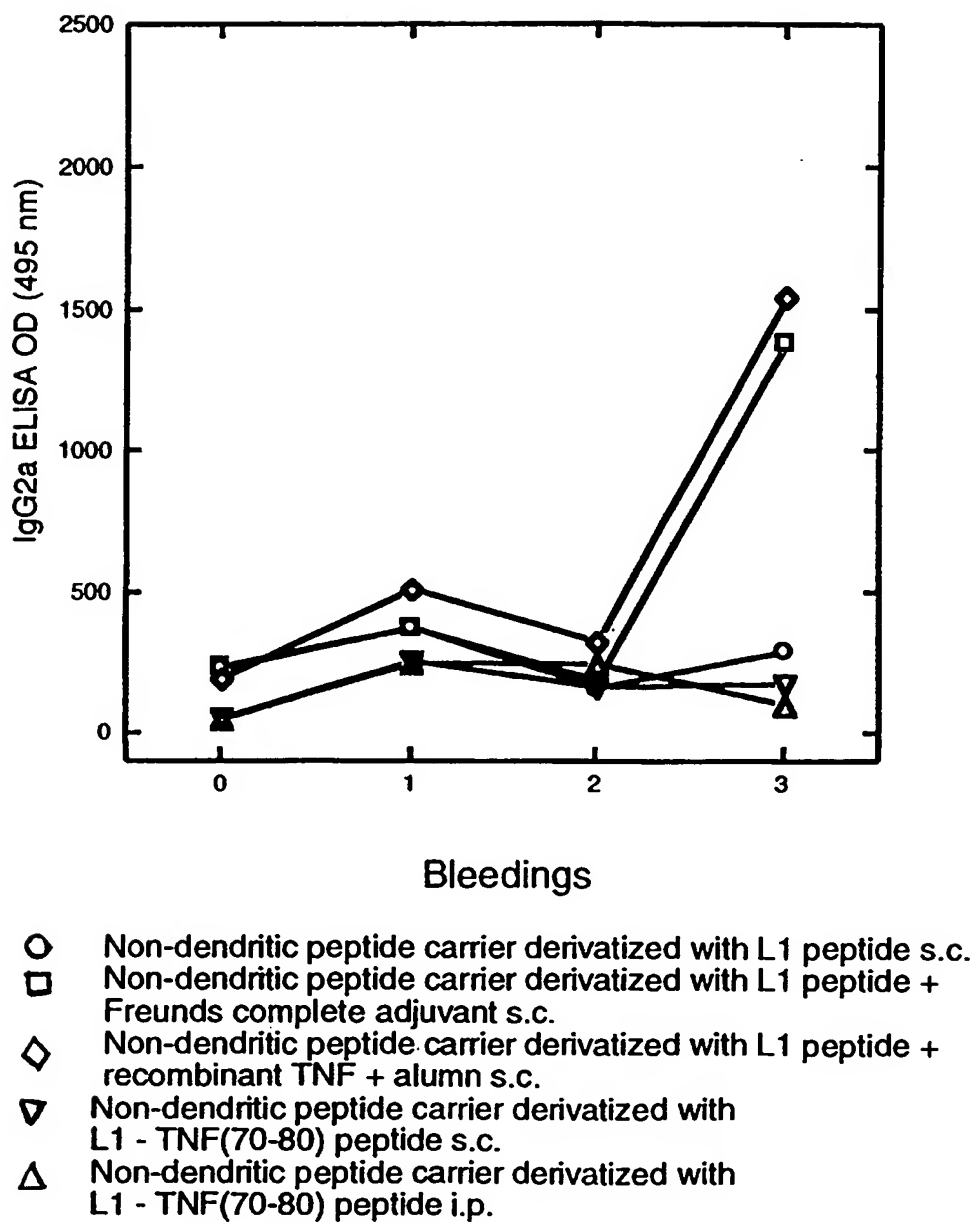   L1 - IFN(1-39) + L1 - IFN(95-133) peptide i.p.

Fig. 31

Fig. 32

Fig. 33

Fig. 34

Bleedings

○ Non-dendritic peptide carrier derivatized with L2 peptide s.c.
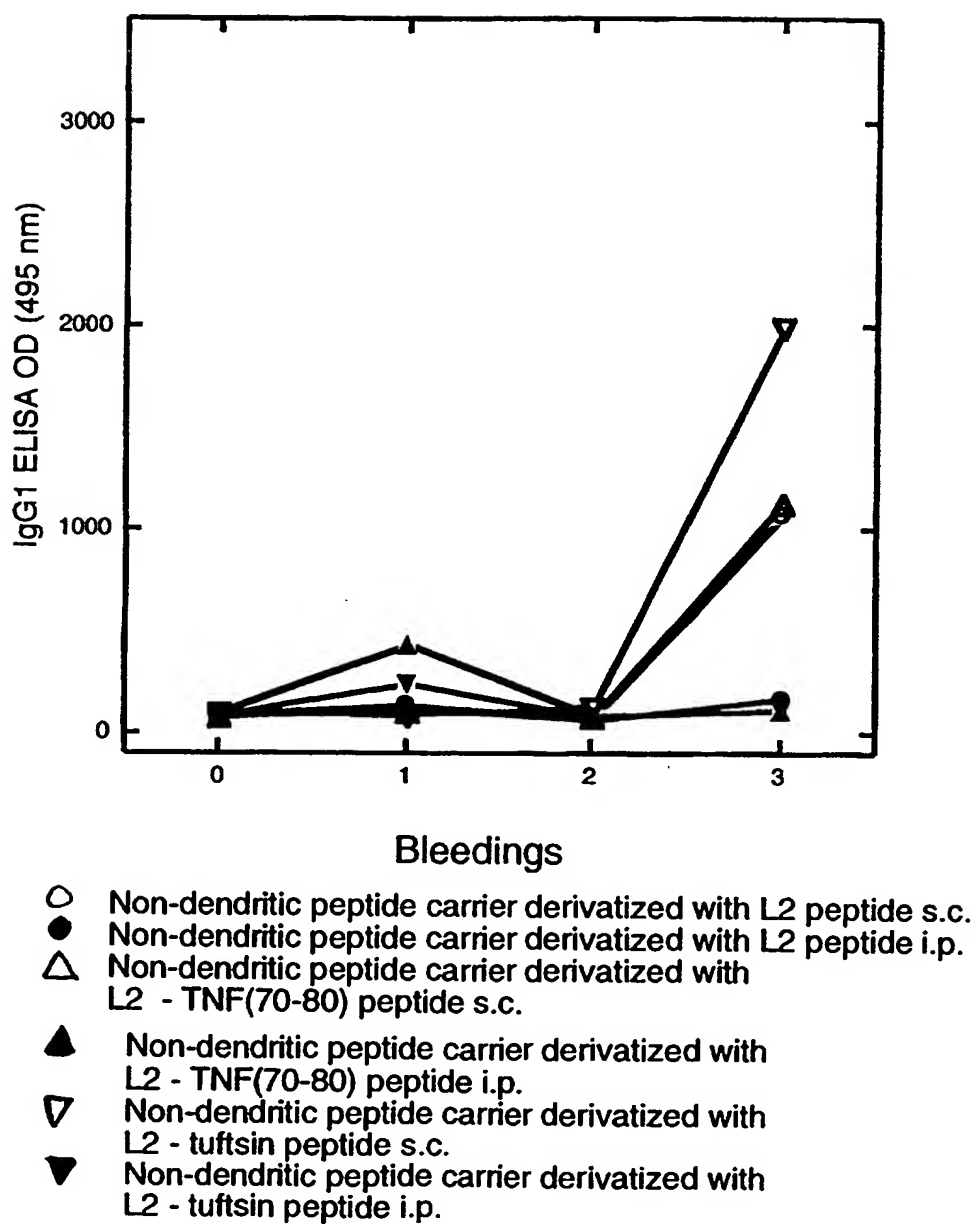● Non-dendritic peptide carrier derivatized with L2 peptide i.p.
△ Non-dendritic peptide carrier derivatized with
    L2 - TNF(70-80) peptide s.c.

▲ Non-dendritic peptide carrier derivatized with
    L2 - TNF(70-80) peptide i.p.
▽ Non-dendritic peptide carrier derivatized with
    L2 - tuftsin peptide s.c.
▼ Non-dendritic peptide carrier derivatized with
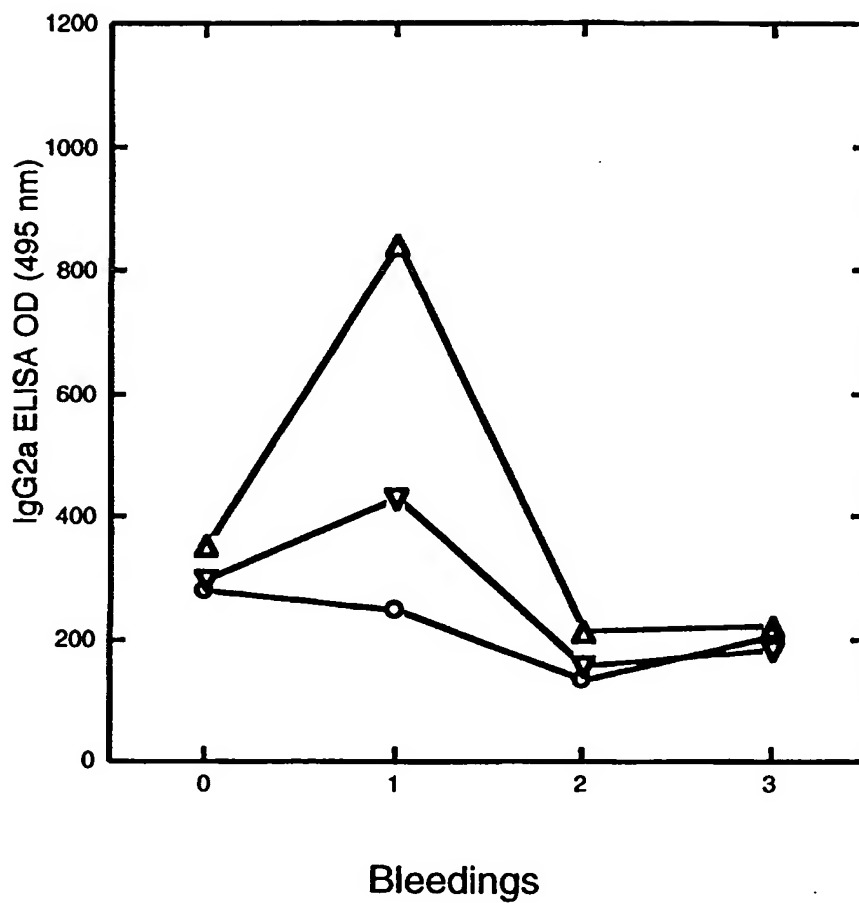   L2 - tuftsin peptide i.p.

Fig. 35

Bleedings

○　Non-dendritic peptide carrier derivatized with L2 peptide i.p.
△　Non-dendritic peptide carrier derivatized with
　　L2 - TNF(70-80) - branch i.p.
▽　Non-dendritic peptide carrier derivatized with
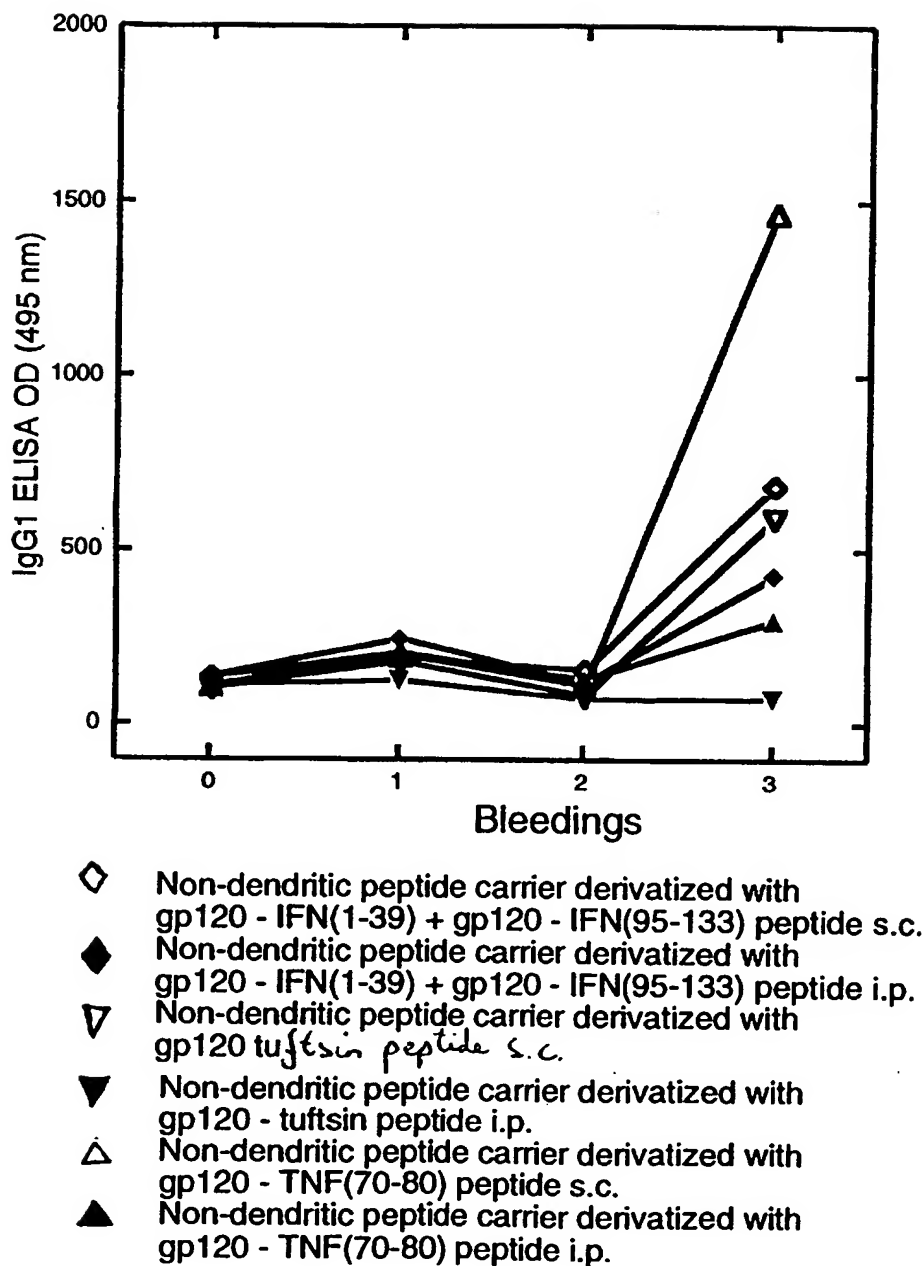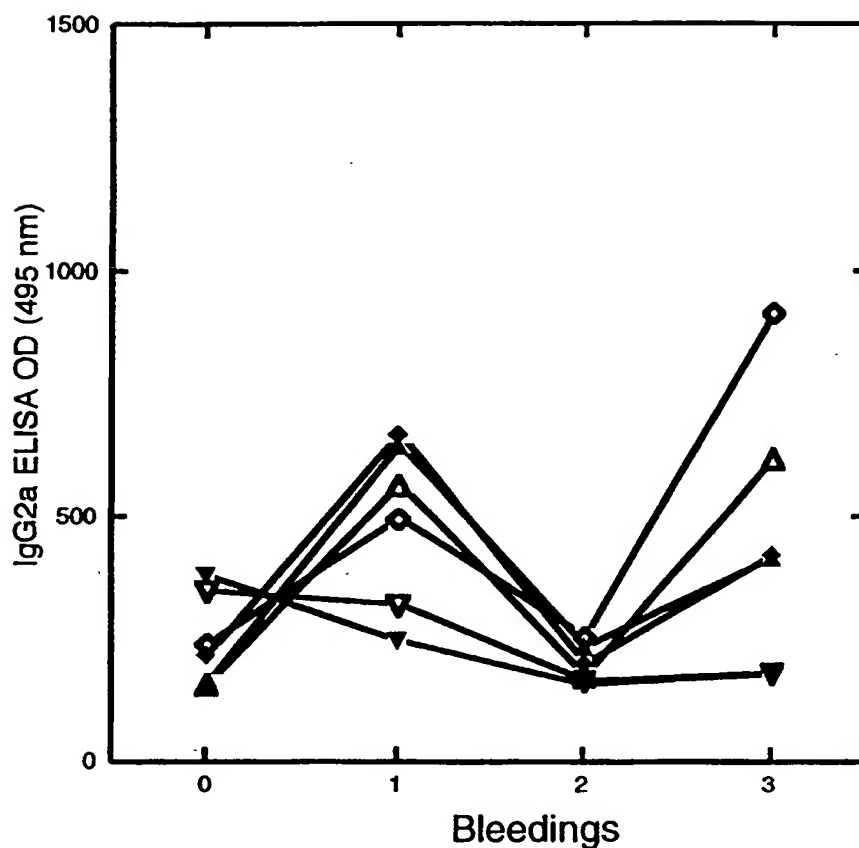　　L2 - tuftsin - branch i.p.

Fig. 36

◇   Non-dendritic peptide carrier derivatized with
gp120 - IFN(1-39) + gp120 - IFN(95-133) peptide s.c.

◆   Non-dendritic peptide carrier derivatized with
gp120 - IFN(1-39) + gp120 - IFN(95-133) peptide i.p.

▽   Non-dendritic peptide carrier derivatized with
gp120 tuftsin peptide s.c.

▼   Non-dendritic peptide carrier derivatized with
gp120 - tuftsin peptide i.p.

△   Non-dendritic peptide carrier derivatized with
gp120 - TNF(70-80) peptide s.c.

▲   Non-dendritic peptide carrier derivatized with
gp120 - TNF(70-80) peptide i.p.

Fig. 37

◇ Non-dendritic peptide carrier derivatized with
    gp120 - IFN(1-39) + gp120 - IFN(95-133) peptides s.c.
◆ Non-dendritic peptide carrier derivatized with
    gp120 - IFN(1-39) + gp120 - IFN(95-133) peptides i.p.
▽ Non-dendritic peptide carrier derivatized with
    gp120
▼ Non-dendritic peptide carrier derivatized with
    gp120 - tuftsin peptide i.p.
△ Non-dendritic peptide carrier derivatized with
    gp120 - TNF(70-80) peptide s.c.
▲ Non-dendritic peptide carrier derivatized with
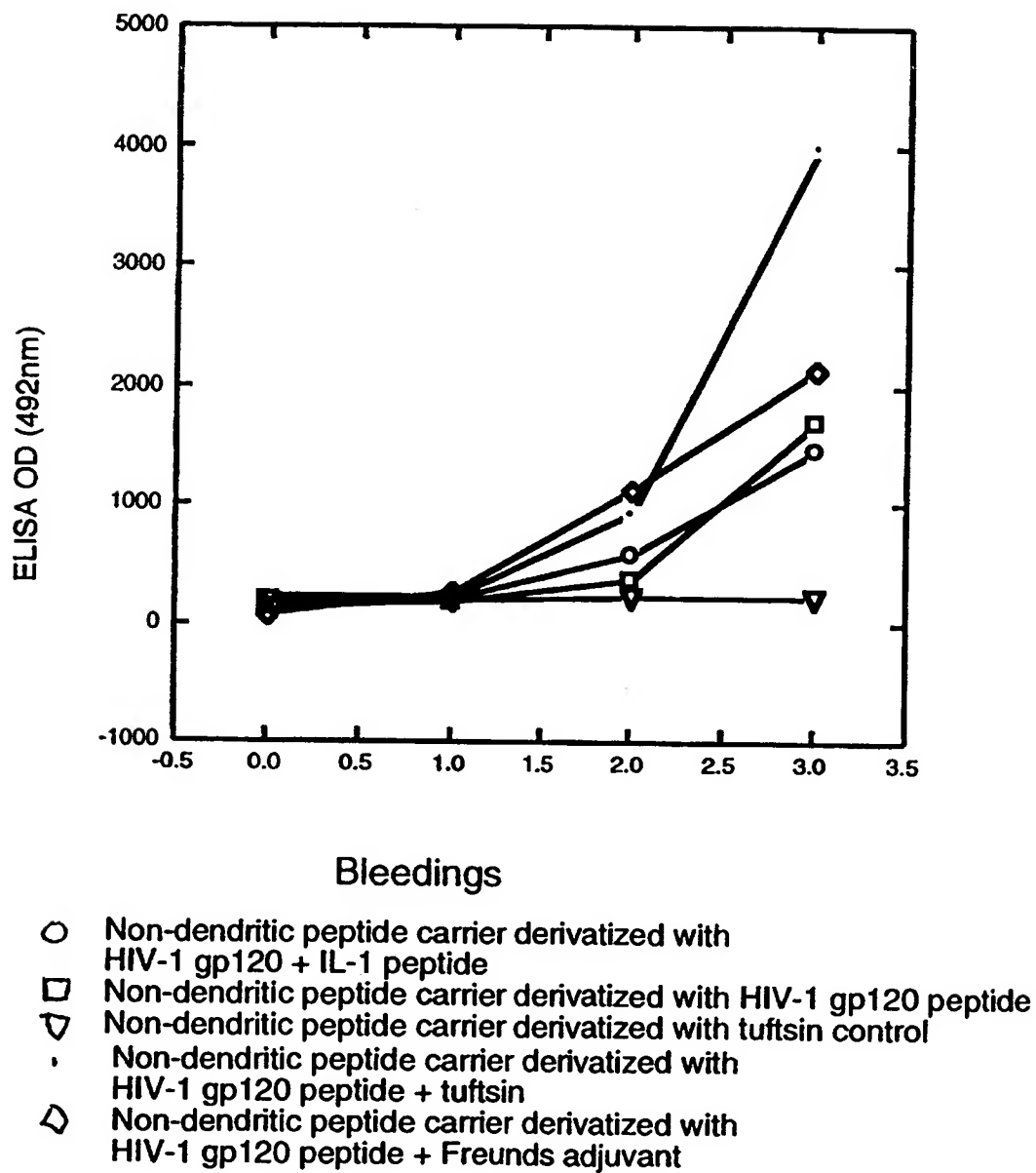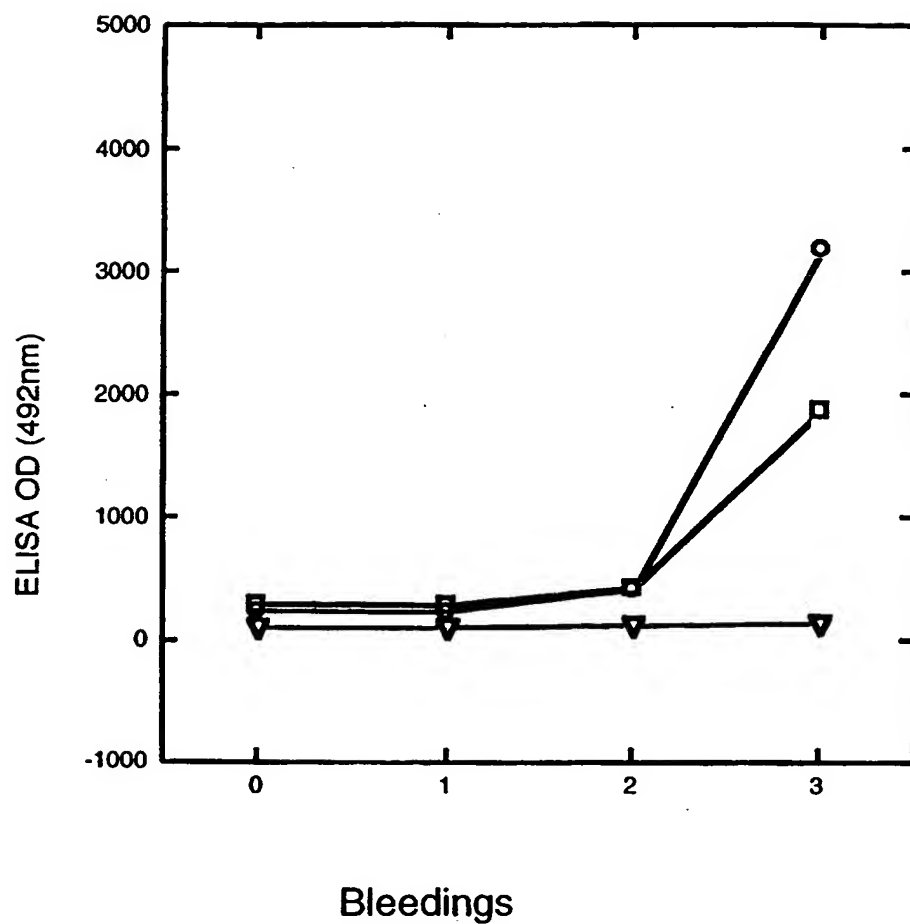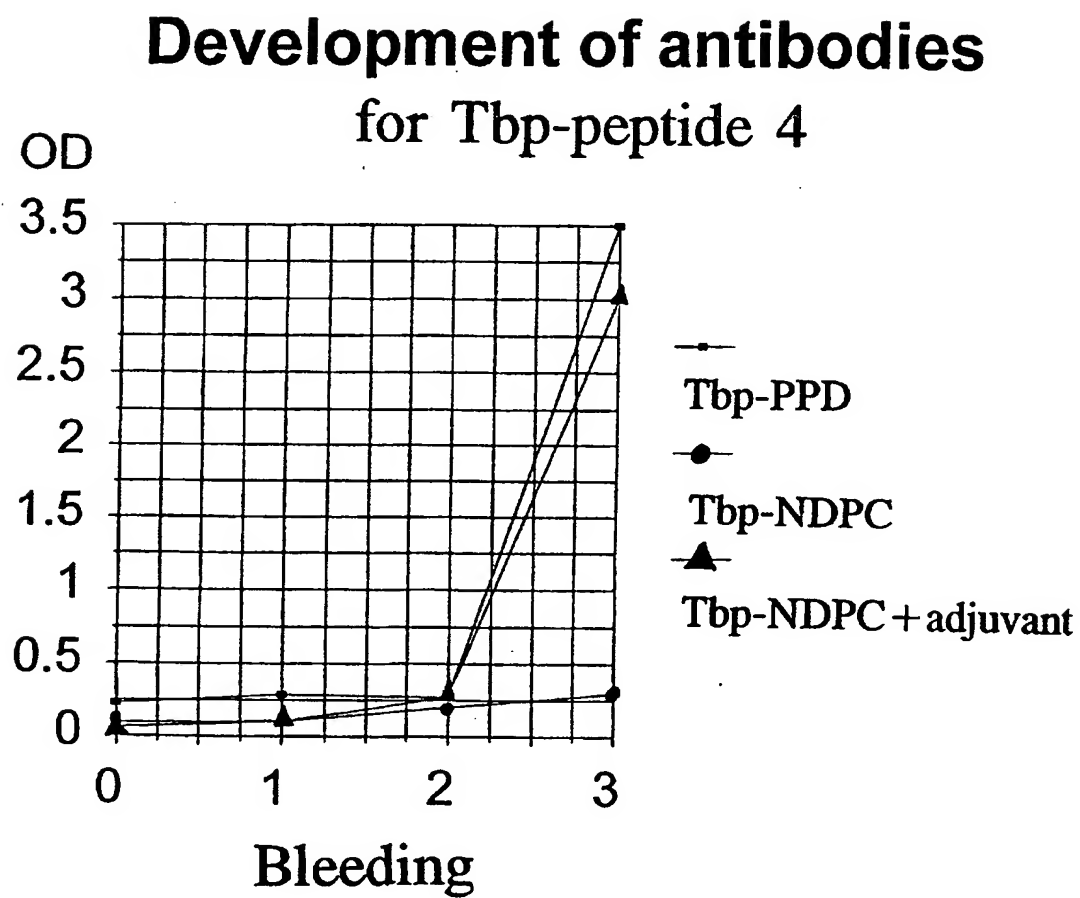   gp120 - TNF(70-80) peptide i.p.

Fig. 38

Bleedings

○ Non-dendritic peptide carrier derivatized with
   HIV-1 gp120 + IL-1 peptide
□ Non-dendritic peptide carrier derivatized with HIV-1 gp120 peptide
▽ Non-dendritic peptide carrier derivatized with tuftsin control
• Non-dendritic peptide carrier derivatized with
   HIV-1 gp120 peptide + tuftsin
◇ Non-dendritic peptide carrier derivatized with
   HIV-1 gp120 peptide + Freunds adjuvant

Fig. 39

Fig. 40

Development of antibodies
for Tbp-peptide 4

Tbp-PPD

Tbp-NDPC

Tbp-NDPC+adjuvant

Fig. 41

Development of antibodies for PalA-peptide 5
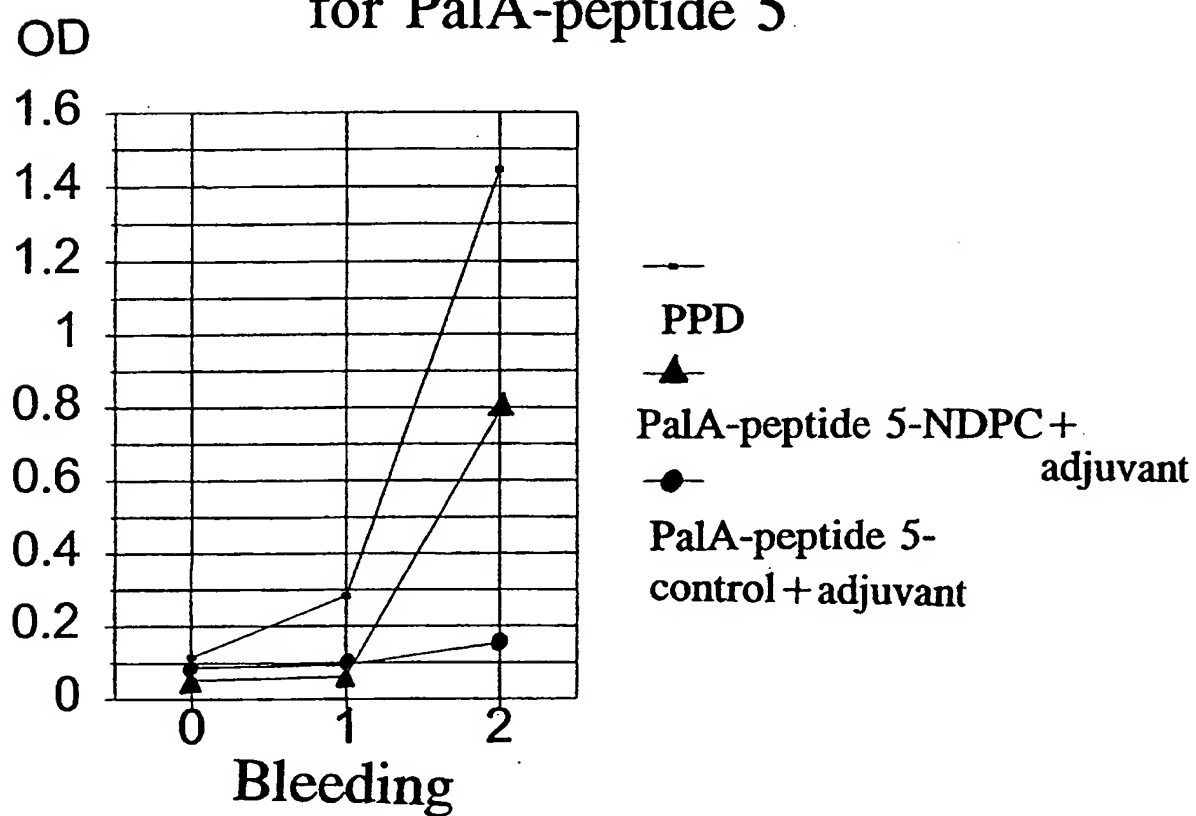
Fig. 42

Influence of different coating peptides on ELISA

Detection Ab is raised against Tbp-PPD
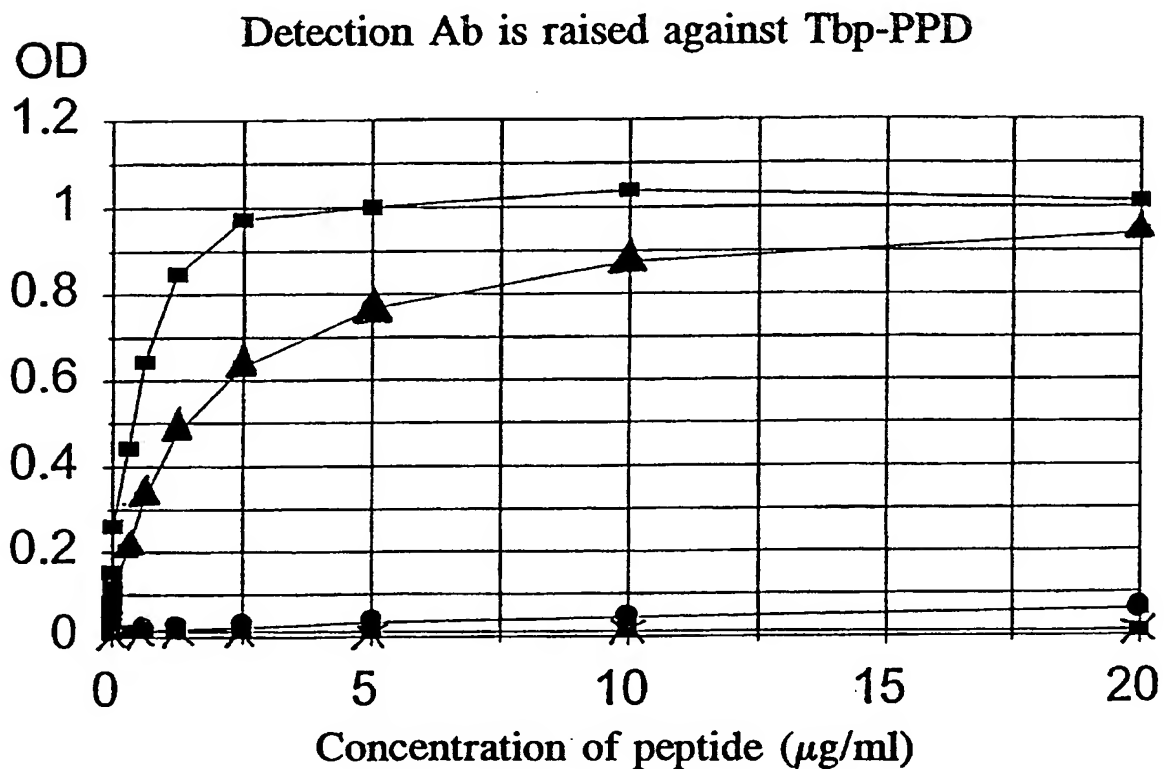
- Tbp-peptide 4 on structure 2 in ISCOM ▬■▬
- Tbp-peptide 4 on structure 3 ▬▲▬
- Palmitoylated Tbp-peptide 4 in ISCOM ▬●▬
- Palmitoylated Tbp-peptide 4 ▬✶▬
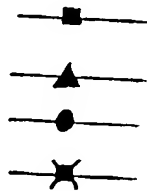
Fig. 43

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K14/00 G01N33/68 A61K38/16 A61K39/385

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 31480 A (SYNTHETIC PEPTIDES INC ;HOUSTON MICHAEL E (CA); ZHOU NIAN E (CA);) 23 November 1995 cited in the application see page 4, line 1 - page 7, line 2 see page 21, line 35 - page 23, line 8; claims; examples ---	1-7, 9-12, 26-30, 43,46, 56-95
X	GB 2 282 813 A (MERCK & CO INC) 19 April 1995 cited in the application see page 7, line 5 - page 13, line 17; claims; examples ---	1,8, 12-15, 56-95
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 July 1997	0 7. 08. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Fuhr, C

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 04543 A (SCRIPPS RESEARCH INST ;DAWSON PHILIP E (US); KENT STEPHEN B H (US)) 16 February 1995 cited in the application see page 5, line 33 - page 8, line 2; claims; examples ---	1,8, 12-15, 56-95
X	WO 94 02506 A (SCHOOL OF PHARMACY UNIVERSITY ;TOTH ISTVAN (GB); GIBBONS WILLIAM A) 3 February 1994 see claims; examples ---	1,2,7,8, 12,13, 15, 20-22, 43-45, 56-95
A	WO 89 10348 A (SYNPHARM LTD ;SANTER VIVIEN BEDFORD (AU)) 2 November 1989 see the whole document ---	1-95
A	WO 95 33766 A (ONTARIO CANCER INST) 14 December 1995 see the whole document -----	1-95

1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00146

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 53,54,66,68-70,80,81 and 93
                is(are) directed to a method of treatment of the human/animal
                body, the search has been carried out and based on the alleged
                effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos..
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

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		AU	3446189 A	24-11-89
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